Ecological functioning of bacterial chitinases in soil

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Chapter 1

General Introduction

Chitin is the second most abundant polymer in nature and it plays an essential role in aquatic and terrestrial ecosystems. In soils, chitin is mainly found in fungal cell walls and in the outer shields, exoskeleton, of arthropods. Because of the relatively high nutritional value of chitin, including both C and N components, many primary decomposers such as bacteria and fungi contain chitin degrading enzymes, among which chitinases.

The possession of chitinases is widely distributed among bacteria (and fungi). As there is a fierce competition between bacteria and fungi in the oligotrophic environment of the soil for the scarce nutrients, bacterial chitinases are also used as tools to attack fungi by destroying the chitin in fungal cell walls. The main target for this activity is the hyphal tip where the chitin is still in a native form, *i.e.* where chitin is present as single strands and not yet cross-polymerized and linked with other cell-wall components, so that it can easily be attacked by chitinases. Previous studies showed that bacteria differ in their ability to use chitinases for chitin degradation (De Boer et al 2008, De Boer et al 1999, De Boer et al 1998). Some are good degraders of solid chitin particles and such bacteria might primarily be involved in the decomposition of dead hyphal (and arthropod) remnants (Igarashi et al 2014). Others can only degrade pre-treated (colloidal) chitin and several of such bacteria have shown to have additional antifungal properties and they may use their chitinases as part of the machinery to compete with or defense against fungi (Aktuganov et al 2008).

Although the importance of bacterial chitinases was recognized in numerous studies there are still many questions about their functioning in nature. A major difficulty in the research on the bacterial use of chitinases is the great variety of occurrence of chitin in polymer complexes in nature including dead and

living fungal hyphae. Until now, most of the studies on bacterial chitinases were performed using chitin resources from the exoskeleton of arthropods. However, chitin of arthropods is different chemically from the chitin of fungal cell wall. Thus, for a deeper understanding of the functioning of bacterial chitinases in soil ecosystems, where fungi are in far greater abundance than arthropods, it is necessary to focus on the chitin from fungi in relation to the particular conditions in which bacteria and fungi share the available niches and compete for nutrients. In this thesis I will provide novel information on the functioning of bacterial chitinases depending on a variety of bacterial-fungal interactions.

Chitin: natural polymers with important ecological function

Chitin is a polymer of *N*-acetyl-D-glucosamine. In 1811, chitin has been described for the first time by the French chemist H. Broconnot. The word "chitin" originates from the Greek word "envelope", which, to some extent, describes its function. It is an important structural compound of the exterior of different organisms, e.g. fungi, crustaceans and insects (Synowiecki & Al-Khateeb 2003). Arthropods (mainly crustaceans) are the major chitin producing organisms in aquatic ecosystems and their annual global chitin production has been estimated to range from 2.8×10^7 T yr-1 for freshwater ecosystems to 1.3×10^9 T yr^{-1} for marine ecosystems (Cauchie 2002). The majority of chitin in terrestrial ecosystems is present in fungal propagules (hyphae, spores etc.) or in remainders of fungi (Smrz & Catska 2010). Although soil arthropods also contain chitin, their biomass and consequently their contribution to the chitin mass in soil is far lower than that of fungi (Holtkamp et al 2008). So far, no reliable estimates exist of the annual fungal chitin production in terrestrial ecosystems. Moreover, it is speculated that also certain bacteria, such as the *Actinobacteria*, among which *Streptomyces* spores, may also contain chitin in the outer sheath (Gomes et al 2008). Other organisms including prokaryotes, plants and vertebrates do not have chitin within their structure (Agullo et al 2003, Keyhani et al 2000).

According to the orientation of the poly-N-acetylglucosamine chains, chitin can be divided into three different forms. The most common form is α-chitin, in which the polymer chains are in antiparallel arrangement. The two other, less common, forms are β- and γ-chitin, in which the polymer chains are in parallel and mixed of parallel and antiparallel arrangements, respectively (Gooday 1990). Crustaceans contain mostly α-chitin; squid pens possess β- chitin. Fungal chitin is comparable to crustacean chitin, due to the uniform composition of the chitinous material, which is both of the alpha-chitin form (Agullo et al 2003).

Chitin is a common structural component of fungal cell walls. It is located in the innermost layer of the cell wall as α-form microfibrils, linked to mannans, glucans and proteins (Figure 1). Generally, the chitin content of fungal cell walls ranges from 22-40 % (Muzzarelli 1977).

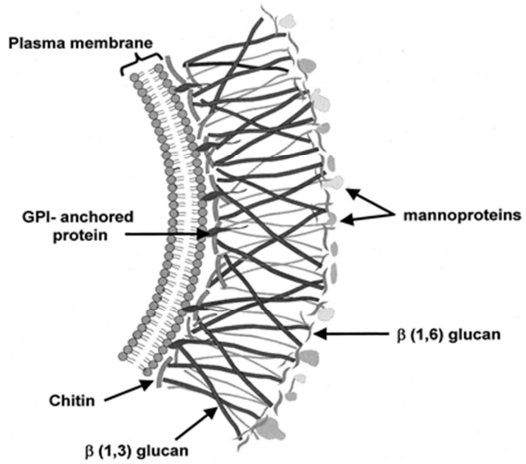

Figure 1 Structure of fungi cell wall (Selitrennikoff 2001)

Chitin has a deacetylated form, called chitosan. Pure chitin rarely exists in nature, instead, natural products may have an acetylation degree of 80%-95% (Kumar 2000). Figure 2 shows the structure of chitin and chitosan.

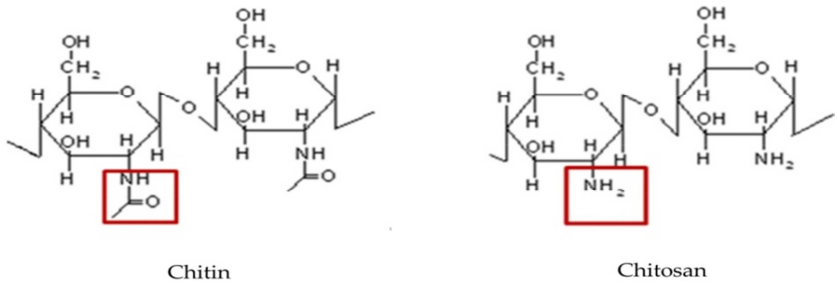

Figure 2 Structure of chitin and chitosan (deacetylation of chitin)

Although the natural forms of chitin are cross-linked with other organic polymers, purified chitin is often used in research, mostly as crystal chitin purified from crab cuticles. To make the chitin more accessible for enzymes, colloidal chitin is prepared from crystal chitin by dissolving it in strong acid followed by precipitation in water (Hirano & Nagao 1988). It is the most used form of chitin to obtain chitinolytic bacteria on agar plates. When chitinolytic bacteria grow on top of colloidal chitin plates, a halo will be produced due to the activity of chitinase (De Boer et al 1998). This method has commonly been used to identify and enumerate chitinolytic bacteria.

Enzymatic Chitin degradation

Chitin is insoluble in water and resistance to moderate acid, alkaline and many organic solvents and therefore microbial degradation is the most important degradation pathway (Hoell et al 2010). Both fungi and bacteria are able to decompose chitin (Ghosh & Chakraborty 2010). In fungi, nearly 80 % of the species are chitinolytic, but it is not clear whether all these fungi do actually degrade chitin since fungal chitinases play also a role in branching of chitin polymers in the fungal cell wall. In different environments 1 – 20 % of culturable bacterial species are chitinolytic (Gooday 1990). The chitinolytic ability is taxonomically widespread among bacteria and can be found in both Gram-negative and Gram-positive genera (Gooday 1990).

Chitin can be degraded enzymatically in two ways. First chitin degradation can be achieved by deacetylation by deacetylases. This degradation will result in the production of chitosan. Through the activity of chitosanase, chitosan can be degraded further into chitobiose and glucosamine.

The main degradation pathway of chitin is soil is through chitinases (EC 3.2.1.14) resulting in the hydrolysis of the 1, 4-β- glycosidic bonds between the N-acetyl-D-glucosamine units. The products of this enzyme activity are monomers of N-acetyl-D-glucosamine (GlcNAc) (Beier & Bertilsson 2013). This may be followed by degradation of the N-acetyl-D-glucosamine by N-acetylglucosaminidases, producing glucosamine.

Bacterial chitinases can be divided into two families according to their amino acid sequence homology: family 18 and 19 of glycoside hydrolases. The majority of the known bacterial chitinases belong to family 18. The catalytic domains of family 18 chitinases, have (β/α)8 barrel folds whereas those of family 19 chitinases have a high α-helical content and share structural similarity with chitosanases and lysozymes (Svitil & Kirchman 1998, Watanabe et al 2001). Glycoside hydrolase family 18 bacterial chitinases can be divided into different types. In 1993, bacterial chitinases were divided into group I to IV, with among group I, a further division of the chitinases in A, B and C types (Watanabe et al 1993). Later on, another classification method was suggested (Karlsson & Stenlid 2008, Suzuki et al 1999), in which the family 18 chitinases was divided in types A, B and C with most of the bacterial chitinases belonging to the A type. This nomenclature is referring to the enzyme catalytic domains (CD). The *ChiA* catalytic domain consists a $(\beta/\alpha)_8$- barrel with a small α+β domain inserted between the seventh and eighth β- strands of $(\beta/\alpha)_8$- barrel (Perrakis et al 1994). Most of the chitinases also contain other domains namely, chitin binding domains (ChBD) and fibronectin type III domains. The ChBD domain regulates the production of a peptide that can bind to chitin. It has been reported to assist in the movement of chitinases along chitin strands and within environments containing chitin (Svitil &

Kirchman 1998). Fibronectin type III domains are thought to maintain an optimal distance and orientation between the catalytic and binding domains (Folders et al 2001). It is reported that chitin binding and fibronectin type III domains are not necessary for the hydrolysis of colloid chitin, but play an important role in hydrolysis of chitin–glucan complex of fungal cell walls (Dehestani et al 2010).

According to their functional patterns, chitinases can also be classified into endo and exo-enzymes (Henrissat & Davies 2000). Endochitinases cleave chitin randomly resulting in chitin-oligomers of different size, such as chitobiose, chitotriose, and chitotetraose; exochitinases include chitobiosidases and β -N-acetylhexosaminidase; the former produces GlcNAc dimers (chitobiose) by cleaving the non-reducing end of chitin while the latter hydrolyzes chitobiose, chitotriose, and chitotetraose, resulting in the release of N-acetylglucosamine (Bhattacharya et al 2007, Tronsmo & Harman 1993).

In order to function, bacterial chitinases need to be associated with the outer parts of bacteria or be secreted as extracellular enzymes (LeCleir et al 2004). The extracellular activity of chitinases suggests that they must be adapted to function under the physicochemical conditions prevailing in the surrounding environment. This may imply that different chitinases will be secreted depending on different situations. Other compounds that cooperate with chitinase during chitin degradation are also secreted by bacteria. For instance, *Serretia marcescens* was found to produce a protein CBP21, which binds to and disrupts the chitin polymer, and so increases the effect of chitinases by making chitin more accessible (Suzuki et al 1998, Vaaje-Kolstad et al 2005a).

Factors affecting the function of bacterial chitinases

The most obvious function of chitinases for bacteria is to enable them to use chitin as a source of energy, carbon and/or nitrogen (Itoi et al 2006, Kishore et al 2005, Mancuso et al 2010). Although many bacteria which are capable of hydrolysing chitin in agar have been isolated, actually little is known about the

ability of these bacteria to degrade fungal chitin resources in soil as the chitin in media is often derived from marine arthropods like crabs and shrimps. Furthermore, chitin in artificial media is often made colloidal to make it more accessible to chitinases. It has been shown that several bacteria that are able to hydrolyse colloidal chitin are very slow degraders of chitin particles (De Boer et al 1999). This observation has led to the suggestion that such bacteria may use their chitinases for different purposes. The bacterial genus *Collimonas* is an example of chitinolytic soil bacteria with limited chitin-degrading abilities (Leveau et al 2010). This bacterial genus is mycophagous, which implies that it uses living fungi as a source of nutrients. It has been proposed that chitinases form part of the machinery with which *Collimonas* attack fungal hyphae. Chitinases could help to destabilize the hyphal tip. Destabilization of the hyphal tip can provide *Collimonas* with the ability to get access to the nutrients in the fungal cytoplasm. In addition to the predatory strategy of *Collimonas,* bacterial chitinases may also be involved in other antagonistic interactions with fungi such as competition and defence against bacteriolysis by fungi (De Boer et al 2005, De Boer et al 1998, Suma & Podile 2013).

The complexity of the bacterial chitinolytic enzyme system is large and there is a large variation in the chitinolytic systems of different bacterial species. In addition, other enzymes, such as β-1, 3-glucosidases, as well as secondary metabolites may be important for the ecological functioning of chitinases. Other cell wall lytic enzymes may be needed to make the chitin of the fungal cell walls accessible to chitinase (Figure 1) (Arlorio et al 1992) and the combination of chitinases and secondary metabolites may be effective to suppress fungal growth. Therefore, it is the combination of chitinases and other enzymes and/or other compounds that may be indicative for a particular ecological function.

To investigate the possibility that chitinases of soil bacteria may have different functions, it is necessary to realize that there are different habitats in soil. A major differentiation in soil compartments is that between bulk soil and

rhizosphere (the soil in the immediate surroundings of the plant root that is under the influence of the roots). In these two compartments, soil characteristics and nutrient resources are different. This is reflected by the composition and distribution of microorganisms. Plant roots secrete exudates such as sugars, organic acids, amino acids, etc, which are energy resources for many microorganisms (Meng et al 2005, Rengel & Marschner 2005). Therefore, in the rhizosphere many bacteria and fungi live and feed on these exudates and other root derived products such as sloughed off root cells (Norton & Firestone 1991). There is a fierce competition between bacteria and fungi for these nutrients (Couteaudier 1992). So in the rhizosphere, bacterial chitinases may primarily be used as tools in the competition between bacteria and fungi (De Boer et al 2008). However, in the bulk soil, bacteria have to grow primarily on dead organic matter among which fungal hyphal remnants containing chitin and here bacterial chitinases may mainly be used to degrade fungal chitin as source of C and N.

Additional factors which may be important for the functioning of chitinases are the morphological characteristics and motility properties of bacteria. There is evidence that hyphal forming bacteria, e.g. *Actinomycetes*, are better able to degrade chitin particles than most non-hyphal bacteria (De Boer et al 1999). *Actinomycetes* do often become the dominant chitin-degrading bacteria when the soil is mixed with solid chitinous material (Krsek & Wellington 2001, Sato et al 2010). The reason for the good chitin degrading ability of *Actinomycetes* might be that the hyphae can penetrate the chitin particles thereby extending the area of activity for chitinases. Next to *Actinomycetes*, bacteria that are highly motile can also be good degraders of chitin particles. Such bacteria may also be able to reach quickly and effectively the interior of chitin particles. A relationship between motility and chitin-degrading ability has been shown for the gliding soil bacterium *Flavobacterium johnsoniae* (Nelson & McBride 2006). Motility hasalso been considered to be an important factor for attack of living fungi by chitinolytic bacteria (Hoppener-Ogawa et al 2009).

Aims and research questions

The research described in this thesis aimed to improve the understanding of the ecological function of bacterial chitinases in soil. The following research questions have been addressed:

1) Is there a relationship between the composition of chitinases genes, as well as other bacterial capabilities, such as the production of antibiotics and fungal cell wall lytic enzymes and the ecological function for which chitinases are used in different habitats?

2) Is there a relationship between the composition of the chitinolytic bacterial community, chitinase genes and the abundance of saprotrophic fungi in situations of competition between bacteria and fungi such as in the plant rhizosphere?

3) What is the response of soil borne bacteria upon the addition of different chitin resources and will there be a selection for different chitinolytic bacteria depending on the type of the available chitin source?

4) What are the main factors in the bacterial chitinolytic system determining the functioning of chitinases, *i.e.* chitin degradation and/or antifungal activity and what are the effects of morphological characteristics, such as hyphaeformation on the functioning of bacterial chitinases?

In order to get a better understanding of the ecological functions of bacterial chitinases and answering above formulated questions, I applied both a "in silico" genomic comparison of the bacterial chitinolytic system and experimental approaches in order to test the hypothesis that the use of bacterial chitinases is affected by the presence and activity of fungi and that there is a link between the presence and activity of bacterial chitinases and of other antifungal tools in bacteria.

Outline of thesis:

In Chapter 2 an “in silico” approach was applied to compare the chitinolytic systems of aquatic and terrestrial bacteria, including chitinase numbers, domains within chitinases and other assisting proteins on the basis of information present in publically available bacterial genomes. These characteristics were connected to possible ecological functions of bacterial chitinolytic systems in different environments.

Chapter 3 provides results of an experiment in which a selection of 13 chitinolytic bacterial species was exposed to different chitin sources. Bacteria were selected from different phyla, having different numbers of chitinases and also differing in morphologic properties. Here, I wanted to find out which of these properties are most important for chitin degradation as well as for the antifungal activity of these bacteria; the latter was tested in confrontation plate assays.

In Chapter 4 I report on the selection of chitinolytic bacteria in soil upon the addition of different chitinous sources, namely crystal chitin, fungal cell walls and exoskeletons of mealworms. The result will reveal effects of chitin resources on soil-borne bacteria.Chapter 5 provides insight in the abundance, composition and diversity of bacterial chitinase genes and the chitinase harboring bacterial community in the rhizosphere as a response to fungal biomass dynamics during potato crop growth in the field.

In Chapter 6, I will discuss the results obtained in this thesis in the view of a broader ecological perspective of the functioning of bacterial chitinases.

Chapter 2

Genomic comparison of chitinolytic enzyme systems from terrestrial and aquatic bacteria

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Environmental Microbiology, 2015, 17, 7

Abstract

Chitin degradation ability is known for many aquatic and terrestrial bacterial species. However, differences in the composition of chitin resources between aquatic (mainly exoskeletons of crustaceans) and terrestrial (mainly fungal cell walls) habitats may have resulted in adaptation of chitinolytic enzyme systems to the prevalent resources. We screened publicly available terrestrial and aquatic chitinase-containing bacterial genomes for possible differences in the composition of their chitinolytic enzyme systems. The results show significant differences between terrestrial and aquatic bacterial genomes in the modular composition of chitinases (i.e. presence of different types of carbohydrate binding modules). Terrestrial *Actinobacteria* appear to be best adapted to use a wide variety of chitin resources as they have the highest number of chitinase genes, the highest diversity of associated carbohydrate binding modules and the highest number of CBM33-type lytic polysaccharide monooxygenases. *Actinobacteria* do also have the highest fraction of genomes containing β -1, 3-glucanases, enzymes that may reinforce the potential for degrading fungal cell walls. The fraction of bacterial chitinase-containing genomes encoding polyketide synthases was much higher for terrestrial bacteria than for aquatic ones supporting the idea that the combined production of antibiotics and cell-wall degrading chitinases can be an important strategy in antagonistic interactions with fungi.

Introduction

Chitin, a polymer of *N*-acetyl-D-glucosamine, is one of the most abundant biopolymers in nature. It is an important structural compound of the exterior of different organisms, e.g. fungi, crustaceans and insects (Synowiecki and Al-Khateeb, 2003). Arthropods (mainly crustaceans) are the major chitin producing organisms in aquatic ecosystems and their annual global chitin production has been estimated to range from 2.8×10^7 $Mgyr^{-1}$ for freshwater ecosystems to 1.3×10^9 Mg yr^{-1} for marine ecosystems (Cauchie 2002). The majority of chitin in terrestrial ecosystems is present in fungal propagules (hyphae, spores etc.) or in remainders of fungi (Smrz & Catska 2010). Although soil arthropods do also contain chitin, their biomass and consequently, their contribution to soil chitin is far lower than that of fungi (Holtkamp *et al.*, 2008). Decomposition of chitin is an important component of carbon and nitrogen cycling in both terrestrial and aquatic ecosystems (Gooday 1990). Bacteria, which do not produce chitin themselves, have an important role in chitin decomposition (Beier and Bertilsson, 2013; Metcalfe *et al.*, 2002). Chitinolytic bacteria occur in both aquatic and terrestrial habitats and produce enzymes, chitinases that hydrolyze chitin to chito-dextrins. Most bacterial chitinases belong to family 18 of the glycoside hydrolases, whereas occurrence of chitinases belonging to the other main chitinase family, glycoside hydrolases 19, is less common (Cantarel *et al.*, 2009). Possession of chitinases is taxonomically widespread among bacteria: chitinolytic representatives are, amongst others, known from the phyla *Actinobacteria*, *Firmicutes*, *Acidobacteria* and *Proteobacteria* (Hunt *et al.*, 2008; Williamson *et al.*, 2000). Yet, the rate and efficiency of chitin degradation appears to differ strongly among chitinolytic bacterial species. *Streptomyces* species were shown to degrade solid chitin particles rapidly and completely in soils and sand microcosms, probably due to their ability to penetrate these substrates with their hyphae (De Boer *et al.*, 1999). On the contrary, many non-filamentous soil bacteria can only decompose chitin efficiently when it is easily accessible (De Boer *et al.*, 1996; De

Boer *et al.*, 1999; Leveau *et al.*, 2010). Therefore, it is uncertain if such bacteria play a major role in the decomposition of natural chitin resources.

A possible alternative function of soil bacterial chitinases is the involvement in antagonistic interactions with fungi (De Boer *et al.*, 2001; De Boer and van Veen, 2001). In soil, co-existence of bacteria and fungi is ubiquitous. Since metabolic abilities of fungi and bacteria overlap, competition for organic resources will occur (De Boer *et al.*, 2005). For instance, competitive interactions between fungi and bacteria for root exudates have received considerable attention as summarized by Buée *et al.* (2009). Thus, chitinases may form part of the interference competitive "tools" of bacteria against fungi. This is supported by the fact that many of the rhizosphere bacterial isolates that are used to prevent root-infection by plant-pathogenic soil fungi possess chitinases (Raaijmakers *et al.*, 2009). Chitinases may also play a role in obtaining nutrients from living fungi. The latter is called bacterial mycophagy and has been examined in detail for soil bacteria of the genus *Collimonas* (Mela *et al.*, 2011). Among all the fungal structures, the hyphal tip is most vulnerable because chitin in this part of the fungal hyphae is not yet linked with glucans and glycoproteins and, therefore, easily accessible to chitinases (Gooday, 1994).In most aquatic habitats fungal biomass is low and saprotrophic fungi make only a minor contribution to aquatic organic matter decomposition (Wurzbacher *et al.*, 2010). Therefore, it is plausible that competitive interactions between bacteria and fungi are far less frequent in aquatic environments than in terrestrial environments. Hence, the aquatic and terrestrial habitats do not only differ in composition of chitin resources (mainly exoskeletons of crustaceans and bryozoans versus mainly fungal cell walls) but also in the intensity of competitive interactions between bacteria and fungi.

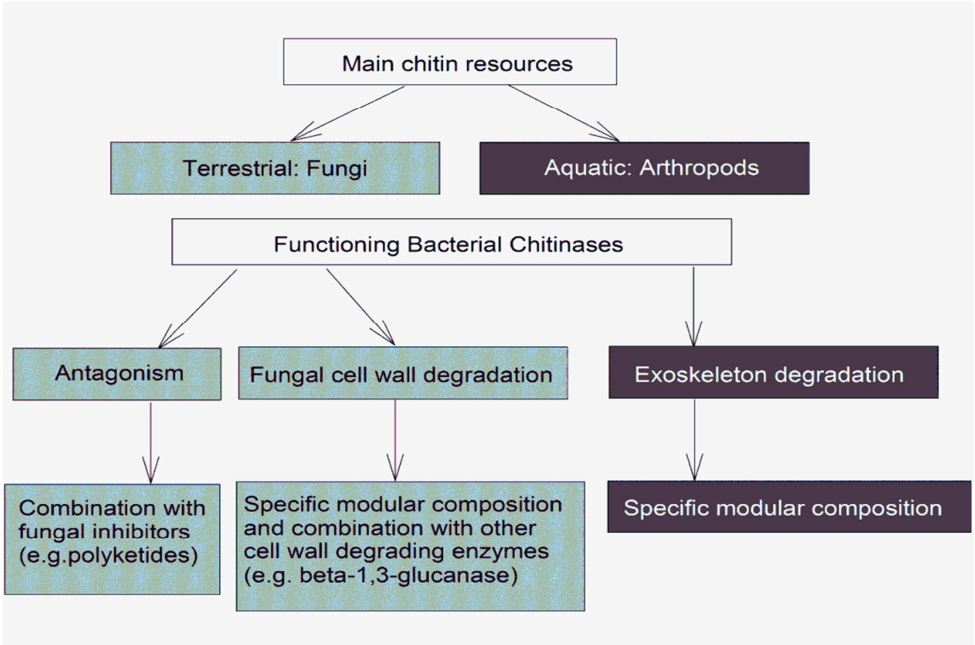

Figure 1 Schematic overview of the hypothesized differences in ecological functioning of chitinases for terrestrial and aquatic bacteria

Based on the aforementioned consideration we hypothesized that the composition of chitinolytic enzyme systems may differ between terrestrial and aquatic bacteria (Figure 1). In particular, we hypothesize that the function of terrestrial bacterial chitinases is strongly related to the decomposition of fungal remainders and/or antagonistic interactions with fungi. To address these hypotheses, we performed an "*in silico*" study by screening and comparing publicly accessible aquatic and terrestrial bacterial genomes with respect to the characteristics of their chitinolytic systems and, at a lower level of detail, with respect to the occurrence of genes encoding other components with possible anti-fungal activities. In addition to addressing our hypotheses, the data presented provide a comprehensive overview of the composition of bacterial chitinolytic enzyme systems, including catalytic hydrolase domains, carbohydrate-binding modules, and the recently discovered lytic polysaccharide monooxygenases (LPMO) (Horn *et al.*, 2012; Vaaje-Kolstad *et al.*, 2010) that are

referred to as CBM33 or, more recently, Auxilliary Activity family 10 (AA10) (Levasseur *et al.*, 2013).

Methods

In order to obtain information on the composition of bacterial chitinolytic enzyme systems, we examined genomes of bacteria that are available in public databases, mainly the NCBI, JGI and GOLD (http://www.ncbi.nlm.nih.gov, http://www.jgi.doe.gov and http://www.genomesonline.org/cgi-bin/GOLD/index.cgi, based on information available in June 2013). The genome protein annotations were screened for the presence of chitinases by searching for the term "chitinase" in the full description of the genome annotation at NCBI websites. Obtained amino acid sequences were first checked for possession of conserved domains characteristic for chitinases using the search tool of The Conserved Domain Database, NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Conserved domain names containing the term "chitinase", including "chitinase-like", "chitinase_glyco_hydro_19" etc., were selected. A further check was done using the Pfam database (http://pfam.sanger.ac.uk) (Punta *et al.*, 2012) in which PF00704 corresponds to family 18 glycoside hydrolases and PF00182 to the family 19 glycoside hydrolases.

Information on the habitats from which the sequenced bacteria were isolated was derived from http://www.megx.net and from the NCBI database, as well as from publications describing the isolation procedure. All information was confirmed by checking related articles manually. We grouped habitats that are distinguished on http://www.megx.net in two categories, namely terrestrial and aquatic. Genomes of isolates from habitats "soil" and "terrestrial" were assigned to terrestrial bacteria; genomes of isolates from habitats "fresh water", "marine water", "aquatic", "sediment" were assigned to aquatic bacteria.

We also examined whether adaptations to aquatic and terrestrial chitin resources may be mirrored by the modular composition of the 18 and 19 chitinases. With respect to the latter, the majority of chitinases contain other domains next to the catalytic domains. In particular domains classified in CAZy as carbohydrate binding modules (CBMs) and fibronectin type III (FnIII) domains. To identify CBMs and relevant FnIII domains (see Vaaje-Kolstad *et al.*, 2013 and below), amino acid sequences of the selected chitinases were submitted to the Pfam database and screened (http://pfam.sanger.ac.uk). Domain names were retrieved from CAZy using the links provided in Pfam.

For identification of chitinases and additional domains, the threshold value for sequence similarity was set to 10^{-6} expect value (E-value). Although usually values smaller than 10^{-20} are considered as good similarity scores, it has been shown repeatedly that comparison of the sequences of functional chitinases may yield higher E-values. For instance, a chitinase from *Ewingella americana* (Inglis *et al.*, 2000), accession number CAA62151.1 in the NCBI database, yields an E-value for the catalytic domain of 2.1^{-7} when searching the Pfam database. Another example is a chitinase from an antarctic *Arthrobacter* sp. strain TAD20 (Lonhienne *et al.*, 2001), accession number CAB62382.1 in NCBI database, which has a functional carbohydrate binding module (CBM_5_12) yielding an E-value of 3.7^{-7} when using the Pfam database.

Genomes encoding confirmed chitinases were further analyzed for the presence of other proteins possibly involved in chitin and/or fungal cell wall degradation. This included the CBM33-type LPMO (PF03067 in the Pfam database), also known as chitin-binding proteins, and currently classified in the CAZy database as AA10 (Auxilliary Activity family 10; Levasseur *et al.*, 2013). The best known member of this family is the 21 kDa chitin-binding proteins CBP21 from *Serratia marcescens* (Vaaje-Kolstad *et al.*, 2005a,b, 2010). Genome annotations were screened by searching for the terms “CBM33” or “chitin binding protein” or “chitin binding domain protein”, obtained sequences were checked using Pfam

(PF03067) applying a threshold of E-value of 10^{-6}. Additional screens were performed, focusing on potential fungal cell wall degrading β -1.3-glucanases and polyketide synthases (PKS) which may produce antifungal secondary metabolites. The full genome annotation texts were screened for the presence of names of these two proteins. The presence of different carbohydrate-binding modules, CBM33 proteins, β -1.3-glucanases and polyketide synthases in chitinase-containing genomes was quantified as the fraction of total chitinase-containing genomes containing at least one of the respective genes. Differences in fractions were statistically analyzed using a Chi square test ($P < 0.05$) after construction of 2 × 2 contingency tables (http://math.hws.edu/javamath/ryan/ChiSquare.html).

Results and Discussion

Phylogenetic distribution of terrestrial and aquatic chitinase-containing bacterial genomes

Of a total of 256 bacterial genomes from terrestrial habitats 74 genomes fulfilled the criteria to be considered as chitinase-containing. For aquatic habitats, we obtained 36 chitinase-containing bacterial genomes out of a total of 401 bacterial genomes. Hence, the fraction of bacterial genomes containing chitinase-genes was much higher for terrestrial bacteria (29 %) than for aquatic bacteria (9 %) (Table 1).

Table 1 Number of publicly available chitinase-containing bacterial genomes from terrestrial and aquatic habitats in June 2013

Habitat	Numbers of chitinase containing bacterial genomes	Total bacterial genomes	Fraction of chitinase-containing genomes
Terrestrial	**74**	**256**	**0.29**
Soil	68	195	0.35
Other terrestrial	6	61	0.10
Aquatic	**36**	**401**	**0.09**
sediment	4	86	0.05
marine	15	229	0.07
Other aquatic	15	58	0.26
fresh water	2	28	0.07

The difference in relative abundance of chitinase-containing genomes between aquatic and terrestrial bacteria could indicate that the possession of chitinase genes is more common for terrestrial bacterial species than for aquatic ones. However, the difference may well be due to a bias in the availability of annotated bacterial genomes. For instance, almost half of the terrestrial chitinase-containing bacterial genomes belonged to the phylum *Actinobacteria* (Table 2). The availability of many annotated genomes of *Actinobacteria* is due to the interest in these microbes as producers of valuable secondary metabolites (Worrall and Vijgenboom, 2010). Earlier studies had already indicated the widespread occurrence of chitinases in terrestrial *Actinobacteria* (Metcalfe *et al.*, 2002; Williamson *et al.*, 2000). The order *Vibrionales* (ɣ-subdivision of the phylum *Proteobacteria*) was strongly represented among the chitinase-containing genomes of aquatic bacteria. The order *Vibrionales* is for the largest part composed of *Vibrio* sp. and *Photobacterium* sp. Their importance in marine chitin degradation has been confirmed repeatedly (Orikoshi *et al.*, 2005; Yu *et al.*, 1991). The reader can find further characteristics of the selected terrestrial and aquatic bacteria and their chitinolytic systems in the online version of this article at the publisher's web-site (doi:10.1111/1462-2920.12545).

Table 2 Taxonomic distribution of chitinase-containing bacterial genomes and average number of chitinases per genome

Phylum	Class/order	Genome numbers in terrestrial habitats	Genome numbers in aquatic habitats	Average numbers of chitinase-genes per genome			
				Terrestrial		Aquatic	
				GH18	GH19	GH18	GH19
Actinobacteria	*Actinomycetales*	29	-	5.38	1.07	-	-
Bacteroidetes		4	3	4	0.25	2.67	0
Firmicutes		22	9	3.45	0.05	2.55	0
	Bacillales	19	5	3.53	0.05	1.6	0
	Clostridiales	3	4	3	0	2.25	0
Proteobacteria		14	24	2.36	0.79	3.67	1
	Beta subdivision	7	1	1.86	0.29	4	1
	Beta/Burkholderiales	7	-	1.86	0.29	-	-
	Beta/Neisseriales	-	1	-	-	4	1
	Delta subdivision	1	1	5	2	1	0
	Gamma subdivision	6	22	2.5	1.17	3.78	1.04
	Gamma/Xanthomonadales	-	1	-	-	2	0
	Gamma/Pseudomonadales	3	-	1.33	1	-	-
	Gamma/Vibrionales	-	15	-	-	4.33	1.27
	Gamma/Oceanospirillales	-	1	-	-	6	1
	Gamma/Enterobacteriaceae	3	1	3.67	1.33	3	2
	Gamma/Alteromonadales	-	4	-	-	1.75	0.25

Phylum	Class/order	Genome numbers in terrestrial habitats	Genome numbers in aquatic habitats	Average numbers of chitinase-genes per genome			
				Terrestrial		Aquatic	
Chloroflexi		1	-	5	0	-	-
Chlamydiae/Verrucomicrobia		2	-	2	0	-	-
Cyanobacteria	*Nostocales*	1	-	1	0	-	-
Deinococcus-Thermus	*Deinococcus-Thermus*	1	-	1	0	-	-

Distribution of family 18 and 19 chitinases

Bacterial chitinases belong to the glycoside hydrolase (GH) families 18 and 19 with the majority belonging to family 18 (Cantarel *et al*., 2009; Henrissat, 1991). The enzymes of these families do not share sequence similarity and have different catalytic mechanisms (Eijsink *et al.*, 2010). Generally, family 18 chitinases are much more abundant in bacteria than family 19 chitinases (Table A1 and A3).

With the exception of three genomes of terrestrial *Mycobacterium spp.* that only contain one single gene encoding a family 19 chitinase, all selected genomes contained family 18 chitinases. The average number of family 18 chitinase genes per selected genome was 3.95 for terrestrial bacteria and 3.14 for aquatic bacteria. The higher numbers in terrestrial bacteria are due to the *Actinobacteria* which have an average number of 5.38 family 18 genes per genome, and in particular to the genus *Streptomyces* that has an average number of 5.82 family 18 genes per genome. Among the aquatic bacteria, the highest numbers of family 18 encoding genes were observed for strains of the order *Vibrionales* (4.31).

Family 19 chitinases are mostly known from plants, but have been detected in several bacterial genera after the first description in 1996 of a family 19 chitinase in *Streptomyces griseus* (Ohno *et al.*, 1996; Prakash *et al.*, 2010). Family 19 chitinases in plants are thought to be involved in defense against plant-pathogenic fungi (Kasprzewska, 2003). Therefore, the possible role of bacterial family 19 chitinases in antagonistic interactions with fungi has received attention. In a comparative study of the antifungal activities of the family 18 and 19 chitinases of *Streptomyces coelicolor*, only a family 19 chitinase showed an inhibitory effect on the extension of fungal hyphae (Kawase *et al.*, 2006). However, other studies have shown that antifungal activity of chitinolytic *streptomycetes* is not restricted to strains possessing family 19 chitinases (Gherbawy *et al.*, 2012).

More generally, it is well known that family 18 chitinases can have anti-fungal activity as well (Prasanna *et al.*, 2013).

The fraction of chitinase-containing bacterial genomes with family 19 chitinases did not differ significantly between terrestrial and aquatic genomes and was 42 % and 50 % (Table A1 and A3), respectively. The number of family 19 chitinases per genome was mostly one, sometimes two, and in three cases three (*Streptomyces* sp. SirexAA-E*, Streptomyces clavuligerus* ATCC 27064 and *Vibrio orientalis* CIP 102891). Most of the terrestrial bacterial genomes containing family 19 chitinases belonged to the *Actinobacteria*, whereas for aquatic bacteria it is mostly found in genomes of ɣ- *Proteobacteria*, in particular *Vibrionales*. With the exception of one *Paenibacillus* genome, the genomes of *Firmicutes* did not contain family 19 chitinases.

Carbohydrate binding modules and fibronectin type III domains within bacterial chitinases

Next to the catalytic domains, most of the chitinases contain other domains, in particular domains classified as carbohydrate binding modules (CBMs) and fibronectin type III (FnIII) domains. It is well known that CBMs increase chitinase affinity for chitin, whereas other functions for CBMs have also been proposed, including facilitation of enzyme movement along a chitin chain during processive action and stimulation of local decrystallization of the substrate (Eijsink *et al.*, 2008; Nimlos *et al.*, 2012; Svitil and Kirchman, 1998; Watanabe *et al.*, 1994). Notably, chitinases acting on heteropolymeric structures such as fungal cell walls may benefit from CBMs that create affinity to other polysaccharides nearby (Hervé *et al.*, 2010). Less information is available for FnIII domains nevertheless a role in enhancement of hydrolysis of chitin has been suggested (Uchiyama *et al.*, 2001; Watanabe *et al.*, 1994).

In order to obtain a better understanding of differences between terrestrial and aquatic bacterial chitinolytic systems, we investigated the variation

in composition of CBMs in chitinases. We found four dominating types of CBMs, with Pfam IDs PF02839, PF03422, PF02018 and PF00553, respectively. Domain PF02839 (CBM5/12 in CAZy) is characterized by the presence of conserved exposed tryptophans that interact with the substrate (Vaaje-Kolstad *et al.*, 2013). This domain is generally thought to be rather chitin-specific and there are reports which confirm its contribution to chitinase efficiency, in particular in the degradation of crystalline chitin (Hashimoto *et al.*, 2000; Uni *et al.*, 2012). Domain PF03422 (CBM6 in CAZy) has been shown to have a cellulose-binding function, whereas some also bind to β -1, 3-glucans, β -1, 4-glucans and xylan (Henshaw *et al.*, 2004; van Bueren *et al.*, 2005). Domain PF00553 (CBM2 in CAZy) has been reported to bind to soluble and insoluble forms of cellulose, whereas binding to xylan and chitin also has been reported (Simpson *et al.*, 1999; Xu *et al.*, 1995). Domain PF02018 covers four CBM families in CAZy (CBM4, CBM9, CBM16 and CBM22) comprising domains known to bind to cellulose, β -glucans, and xylan (Brun *et al.*, 2000; Winterhalter *et al.*, 1995).

Chitinases contain two families of FnIII domains. One of the families, PF08329, includes the N-terminal domain of the well-known chitinase A from *S. marcescens*, which contains exposed aromatic residues that were found to enhance the hydrolysis of chitin (Uchiyama *et al.*, 2001). The role of the other FnIII domain, PF00041, is not clear. The limited available information indicates that this domain plays a functional role in the hydrolysis of chitin but is not directly involved in the binding to chitin (Watanabe *et al.*, 1994). For this latter domain, a more structural role, e.g. in ensuring correct positioning of other domains relative to each other, has been proposed (Watanabe *et al.*, 1994).

From the selected 74 terrestrial and 36 aquatic chitinase-containing bacterial genomes, 292 and 113 family 18 chitinases were obtained, respectively. Of the terrestrial family 18 chitinases 76 % contained at least one CBM domain, whereas for the aquatic family 18 chitinases this fraction was 72 %.

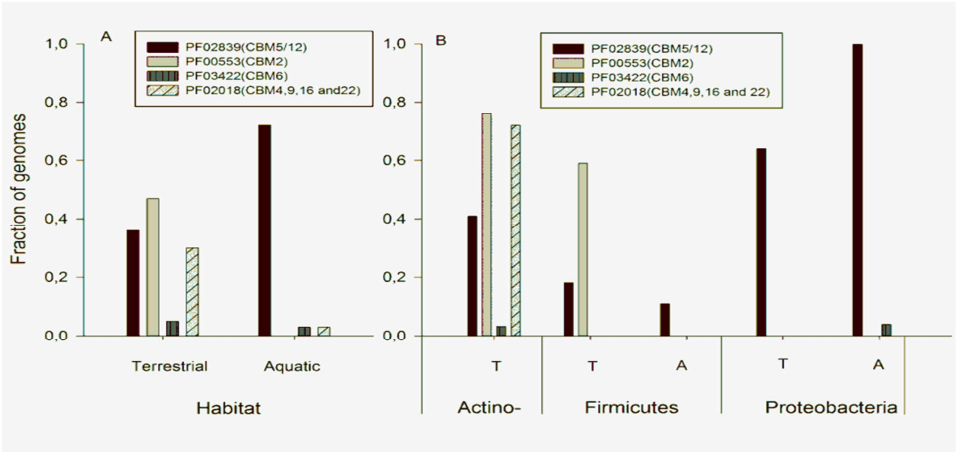

Figure 2 Fraction of bacterial genomes that contain at least one of the indicated carbohydrate binding modules in a chitinase gene. (A) For all genomes; (B) For selected phyla (only those for which a larger number of chitinase-containing genomes were available; Actino-: *Actinobacteria*; T: terrestrial habitat; A: aquatic habitat)

Figure 2 showed some interesting trends. The chitin-specific CBM5/12 domain (PF02839) commonly occurs in chitinases of all bacterial phyla and its occurrence is significantly higher in genomes derived from aquatic habitats than in terrestrial genomes ($p < 0.05$). A small number of CBM5/12 domains group in another Pfam entry (PF14600, referred to as CBM_5_12_2). These CBMs only occurred in bacterial chitinases from aquatic *Proteobacteria* (Table A3). Most interestingly, aquatic bacteria hardly contained any other CBMs, whereas many additional CBMs occur in the chitinases of terrestrial bacteria. CBM2 (PF00553) is abundant in the terrestrial bacterial chitinases, where they, according to available exprimental data, may contribute to binding of chitin, but also could interact with other polysaccharides. The other CBMs found in terrestrial chitinases, belonging to CBM families 4, 6, 9, 16 and/or 22 (PF02018 and PF03422), are not likely to bind to chitin but rather to β -glucans. These domains, only found in chitinases from actinobacterial genomes, may endorse the chitinases with the ability to interact with β -glucan containing co-polymeric structures which are present in fungal cell walls (Bowman and Free, 2006). The general impression of the distribution of CBMs among chitinase-containing genomes is that the degradation

of chitin requires more complex modular chitinase architectures in terrestrial habitats than in aquatic habitats. This might be a general adaptation to larger variation in substrate composition or a more specific adapatation to the occurrenc of fungi.

Some chitinases from terrestrial *Actinobacteria* and *Proteobacteria* contained a CBM13 domain, which have Pfam IDs PF00652 or PF14200, and are named Ricin_B_lectin and RicinB_lectin_2, respectively. Both of them are related to a lectin (a carbohydrate-binding protein) produced in the seeds of the castor oil plant *Ricinus communis* (Lord *et al.*, 1994). There is no evidence that this domain is involved in the functioning of chitinases. Due to their unknown function and limited numbers, PF00652 and PF14200 were not included in our analysis.

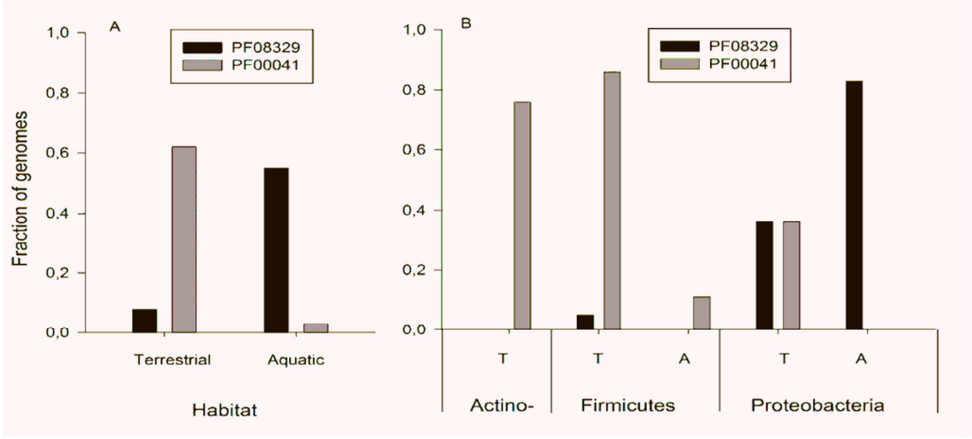

Figure 3 Fraction of bacterial genomes that contain one or more of the indicated fibronectin type III domains in at least one of their chitinases (only family 18 enzymes) (A) for all genomes (B) for selected phyla (only those for which a larger number of chitinase-containing genomes were available; Actino-: *Actinobacteria*; T: terrestrial habitat; A: aquatic habitat)

FnIII domains PF00041 and PF08329 are present in chitinases originating from both terrestrial and aquatic habitats. However, the FnIII domain PF00041 is far more frequent in chitinases of terrestrial bacterial genomes (62 % of genomes) than in aquatic genomes (3 %) (Figure 3). In particular, this domain is highly represented in chitinases encoded by genomes of the terrestrial phyla

Actinobacteria and *Firmicutes* and, to a lesser extent, *Proteobacteria.* The prominent presence of the FnIII domain PF00041 in terrestrial bacterial chitinases may point at a role in the degradation of fungal chitin resources. As mentioned before very little is known about the function of PF00041; if this domain indeed has a structural role (see above) its presence in chitinases from terrestrial origin may be linked to the higher modular complexity in these enzymes, relative to those from aquatic habitats. The other FnIII domain, PF08329, most likely being directly involved in chitin-binding, is highly present in bacterial chitinases of aquatic bacteria (Figure 3), in particular in aquatic *Proteobacteria* (around 80%). Consequently, in aquatic habitats, PF08329 is the more common FnIII domain within bacterial chitinases, whereas PF00041 is the by far most common FnIII domain in chitinases from terrestrial habitats. Further research on the functioning of these domains, especially the PF00041 domains, is warranted.

The presence of FnIII domains was restricted to family 18 chitinases. None of the family 19 chitinases contain FnIII domains. The only CBM present in family 19 chitinases is CBM5/12 (PF02839). CBM5/12 domains are particularly frequent in family 19 chitinases of aquatic *Vibrionales* (71 %) (Table A3).

Occurrence of lytic polysaccharide monoxygenases- CBM33 proteins

We screened the chitinase-containing genomes for the presence of genes encoding other enzymes that could contribute to the functioning of chitinases in chitin-degradation or in antifungal activity. First, we examined the presence of CBM33 proteins which today are known as lytic polysaccharide monooxygenases (LPMOs) and which have been reclassified in CAZy as Auxilliary Activity family 10 (Horn *et al.,* 2012; Levasseur *et al.*, 2013). These proteins are known to contribute to the efficiency of chitinases (Vaaje-Kolstad *et al.*, 2005a) and they do so by catalyzing oxidative cleavage of glycosidic bonds in crystalline chitin. This reaction, which depends on molecular oxygen and an electron-donor, introduces chain breaks in the polysaccharide chains without the need of first “extracting” these chains from their crystalline matrix and promotes further degradation by

chitinases (Horn et al., 2012; Vaaje-Kolstad *et al.*, 2010). In addition, CBM33s from *Streptomyces tendae* Tu901 and *Bacillus thuringiensis* are known to possess antifungal activity (Bormann *et al.*, 1999; Mehmood *et al.*, 2011).

Our analyses showed that genes encoding CBM33s are widely distributed among chitinase-containing bacterial genomes. The fraction of chitinase-containing genomes that contained CBM33s did not differ significantly between terrestrial (74 %) and aquatic (61 %) genomes (Table 3). CBM33s are monooxygenases and require oxygen for their functioning (Vaaje-Kolstad *et al.*, 2010). Accordingly, none of the genomes of strictly anaerobic bacteria contained CBM33s (Table A2 and A4).

Occurrence of other proteins possibly related to anti-fungal activity

Next to chitin, fungal cell walls contain different polysaccharides, such as β -1, 3-glucan. Therefore β -1, 3- glucanases are involved in the degradation of fungal cell walls and may provide chitinolytic bacteria with an additional defense mechanism against fungi (Aono *et al.*, 1992; Arora *et al.*, 2007; Hoshikawa *et al.*, 2012). Yet, the frequency of β -1, 3- glucanases was low in both aquatic and terrestrial chitinase-containing bacterial genomes with the exception of genomes of terrestrial *Actinobacteria* (Table 3). Several studies have suggested the importance of β -1, 3- glucanases in antagonistic interactions of bacteria against fungi (Shi *et al.*, 2010). However, other studies did not find relationships between antifungal activity of chitinolytic bacteria and the production of β -1, 3- glucanases (De Boer *et al.*, 1998). In addition, chitinolytic terrestrial bacteria with known antifungal activity, such *as Collimonas fungivorans* and *Serratia plymuthica*, do not have β -1, 3- glucanase-encoding genes (Leveau *et al.*, 2010). Therefore, there is not much evidence that β -1, 3- glucanases are an important component of the antifungal arsenal of bacteria. Instead, the prominent presence of β -1, 3-glucanases in chitinolytic *Actinomycetes* suggests that they may be important in the degradation of remainders of dead fungi which consist mainly of cell walls.

Bacterial polyketides are secondary metabolites that often have antifungal activities (Staunton and Weissman, 2001; Zirkle *et al.*, 2004). Polyketide synthases (PKSs) are a family of multi-domain enzymes or enzyme complexes that are involved in the production of these secondary metabolites. The fraction of PKSs positive bacterial genomes was significantly higher for terrestrial bacteria (81 %) than for aquatic ones (39 %) (Table 3). Therefore, PKSs do appear to be more common for terrestrial chitinolytic bacteria. This observation supports the assumption that the combination of chitinases and antibiotics may be important in antagonistic activities of soil bacteria against fungi (De Boer *et al.*, 2001; De Boer and van Veen, 2001). Interestingly, it has been shown that induction of chitinase gene expression coincided with upregulation of genes encoding secondary metabolites in *Streptomyces* (Świątek *et al.*, 2012; Nazari *et al.*, 2013).

Table 3 Fraction of chitinase-containing bacterial genomes containing at least one gene encoding CBM33-type LPMOs, β-1, 3-glucanase or polyketide synthase

Phylum	Habitat	Fraction		
		CBM33	β-1,3-glucanase	Polyketide synthases
Total bacterial genomes	terrestrial	0.74	0.20*	0.81*
Total bacterial genomes	aquatic	0.61	0.08	0.39
Actinobacteria	terrestrial	0.86	0.41	0.90
Firmicutes	terrestrial	0.86*	0.09*	0.68*
	aquatic	0.22	0.00	0.22
Proteobacteria	terrestrial	0.71	0.00*	0.79*
	aquatic	0.83	0.08	0.42

*: significant difference between terrestrial and aquatic habitat according to chi square test (P< 0.05)

Conclusions and perspectives:

The occurrence of multiple chitinases is thought to reflect the flexibility of bacteria to deal with variability in chitin structures, e.g. parallel (α) and anti-parallel (β) orientations of chitin-chains and different degrees of acetylation (Hunt *et al.*, 2008; Svitil *et al.*, 1997). In addition, synergistic actions of different chitinases in bacteria have been reported (Horn *et al.*, 2006; Techkarnjanaruk and

Goodman, 1999). Hence, the numbers of chitinases in a bacterium could affect the efficiency of its chitin degradation ability (Beier and Bertilsson, 2013). Our study reveals clear differences in the complexities of bacterial chitinolytic machineries regardless of the difference between terrestrial and aquatic ecosystems.

Our screening of the modular composition of chitinases in bacterial genomes indicates differences between aquatic and terrestrial bacteria that could be related to the different chitinous resources they utilize. Arthropods exoskeleton and fungal cell walls form the major source of chitin in aquatic and terrestrial habitats, respectively. Whereas both arthropod exoskeletons and fungal cell walls can be considered as multiphase composite material, their composition is very different. In arthropod exoskeletons α-chitin fibers are embedded in a matrix of proteins that can also contain minerals, such as calcite (Moussian *et al.*, 2005; Raabe *et al.*, 2006). The composition of fungal cell walls is completely different. The latter are mainly composed of α-chitin, glucans, mannans and glycoproteins with chitin, glucans and glycoproteins being covalently cross-linked (Bowman and Free, 2006). In addition, the ultrastructure of chitin fibres differs between arthropods and fungi (Bowman and Free, 2006; Raabe *et al.*, 2006). It is to be expected that such differences between arthropod exoskeletons and fungal cell walls are reflected in adaptations of bacterial chitin-degrading enzyme systems. The present study indeed reveals such adaptations. In particular, whereas the CBMs connected to chitinases from aquatic bacteria seem to be targeting chitin only, chitinases from terrestrial bacteria, in particular *Actinomycetes*, have a CBM repertoire indicating binding to composite polysaccharides, in particular the β -glucan-chitin complexes present in fungal cell walls. More research is needed to elucidate the specific roles of the different chitinase-associated CBMs and FnIII domains in degradation of chitin in arthropod exoskeletons and fungal cell walls.

The high abundance of polyketide synthases in chitinase-containing genomes of terrestrial *Firmicutes* and *Proteobacteria* comparing to aquatic ones may indicate that several non-filamentous terrestrial bacteria use a combination of chitinases and antibiotics in antagonistic interactions with fungi. In addition, the low abundance of β -1, 3-glucanases in genomes of these groups of non-filamentous bacteria does point at the inability to degrade the glucans in the fungal cell walls that are cross-linked with chitin polymers. This cross-linking has not yet occurred in the hyphal tip of fungi, which are therefore most vulnerable to destabilization by chitinases and which may be the prime target for *Firmicutes* and *Proteobacteria*.

An issue of great interest is the role of the recently discovered LPMOs, which, in bacteria, are of the CBM33-type. Their role in chitin-degradation is indisputable but their possible effect as an anti-fungal agent remains largely unexplored. Certain bacteria, such as *Streptomycetes* contain several CBM33-type LMPOs and it is conceivable that these have different substrate specificities (Horn *et al.*, 2012). They might act on different substrates (e.g. chitin and cellulose) (Forsberg *et al.*, 2011), on different forms of the same substrate (e.g. α-chitin vs β-chitin), or on the same substrate in different composite materials. This is a novel area of research and so far only few CBM33-type LPMOs have been experimentally characterized.

It should be noted that our screening of chitinase-containing genomes has several limitations. For instance, for both the terrestrial and aquatic chitinase-containing genomes there is an over-representation of certain bacterial groups (*Actinomycetales* in terrestrial habitats and V*ibrionales* in aquatic habitats). In addition, as we included genes annotated as “chitinase-like” and “chitinase-like super family”, there is a possibility that some of the enzymes counted as chitinases are not functional. On the other hand, it is possible that a few functional chitinases have been excluded. One example is the functional chitinase from *Collimonas fungivorans* Ter331 (accession number: YP_004753623.1). It has

an E-value exceeding 10^{-6} and has been excluded from the current research. Furthermore, bacteria which are difficult to culture are not present in this paper because of the lack of publicly available genomes. Despite these minor errors, our analyses provide an important first step to unravelling the compositions of and the differences between terrestrial and aquatic chitinolytic systems, which again are linked to different degradative capabilities and ecological roles. Our data reveal clear trends in how chitinases in different habitats are composed. The observed differences in modular structure and the rather clear correlations of the occurrence of certain CBMs and FnIII domains with bacterial habitats give directions for further studies on the functional roles of these domains.

In conclusion, our “in silico” screening of chitinase-containing bacterial genomes has indicated possible ecological relevant differences in the functioning of chitinases between terrestrial and aquatic bacteria. Experimental studies are needed to lend further support to the proposed differentiation in ecological functioning of bacterial chitinases.

Chapter 3

Chitin degrading abilities and antifungal activity of bacterial strains differing in complexity of their chitinolytic systems and growth form

Yani Bai, Johannes A. van Veen, Wietse de Boer

Abstract

Chitinolytic bacteria have different abilities with respect to chitin degradation and antifungal properties. This might be partially explained by differences in numbers of chitinases and/or possession of additional enzymes. In order to get a deeper understanding of the factors determining the use of chitinases for chitin degradation and antifungal activity, we selected 13 bacteria from different phyla and tested their chitin degrading ability in liquid cultures and the antifungal activity on agar plates. Next we compared the degradation rates and antifungal activity with the composition of the chitinolytic system of the strains as based on their annotated genomes. Degradation rates of chitin resources differed strongly between bacteria. The results supported the recently suggested importance of chitin-binding proteins in degradation of crystalline chitin as numbers of chitin-binding proteins on genomes rather than numbers of chitinases correlated positively with degradation of chitin particles. Although hyphal growth form has been indicated as an important trait for degradation of crystalline chitin, we found strong variation in the degradation abilities of both filamentous and non-filamentous bacteria. There were no clear indications for a relationship between the composition of the chitinolytic system and antifungal activities of the strains.

Introduction

Chitin is one of the most abundant biopolymers in nature (Chater et al 2010, Gooday 1990). Chitin degradation, which is mainly a microbial process, is important in global carbon and nitrogen cycling. Chitinases are the major chitin decomposing enzymes (Bhattacharya et al 2007). Bacterial chitinases are typically divided into two families according to their amino acid sequence homology: family 18 and 19 of glycoside hydrolases (Henrissat & Davies 2000). The majority of the known bacterial chitinases belong to family 18. Chitinases of both families have also been reported to exhibit antifungal activity (Kawase et al 2006, Prasanna et al 2013).

The ability to hydrolyze chitin is taxonomically widespread in bacteria and is found for both Gram-negative and Gram-positive genera (Gooday 1990). Yet, bacteria differ strongly in their ability to degrade chitin. This has been ascribed to differences in chitinolytic systems, i.e. different numbers of chitinases and different types of chitinases (Bai et al 2015). In addition, Vaaje-Kolstad et al (2005a) showed that chitin binding proteins, also called CBM33 proteins, contribute to the efficiency of chitinases. These proteins were reported to catalyze oxidative cleavage of glycosidic bonds in crystalline chitin, thereby making the chitin fibers more accessible for chitinases (Vaaje-Kolstad et al 2005b). Hence, these proteins can contribute to the chitinolytic performance of bacteria.

Furthermore, next to physiological properties also morphological properties can influence chitin degrading capabilities. Hyphal forming bacteria, such as *Actinomycetes*, are reported to be better capable of degrading chitin particles than most non-hyphal bacteria (De Boer et al 1999). Filamentous bacteria were often found to be the dominant chitin-degrading bacteria when chitinous material was added to soil (Krsek & Wellington 2001, Sato et al 2010). One of the possible reasons for the good chitin degrading abilities of *Actinomycetes* might be that the hyphae can penetrate the chitin particles

thereby extending the area where chitinases are in close contact with the chitin polymers.

The overall hypothesis of this study is that the chitin degrading performance and antifungal activity of chitinolytic bacteria is determined by the number of chitinases (including family 18 and 19 chitinase), the number of chitin binding proteins and the bacterial growth form. In the current study this was tested using a selection of 13 bacterial chitinolytic bacterial strains for which genome information is available. These bacteria were exposed to different forms of chitin in liquid media to test their chitin degrading abilities. In addition, their antifungal activity capabilities were tested in an *in-vitro* confrontation assay with different fungi. The aim of the study was to indicate the possible importance of the chitinolytic system complexity and morphological characteristics for bacterial chitin degrading capacities and antifungal activities.

Method

Strains

We selected 13 chitinolytic bacterial strains that had been isolated from soil and for which genome information is publicly available (National Center for Biotechnology Information database: http://www.ncbi.nlm.nih.gov). These bacteria are from different phyla, have different number of chitinases and have different morphological characteristics (hyphal *versus* unicellular). The selected strains are listed in Table 1.

Table1 Characteristics of the selected bacterial strains

Strain name	Gram	Order	Chitinase number		Filamentous	Chitin binding proteins number
			family 18	family 19		
Streptomyces coelicolor A3 (2)	+	Actinomycetales	8	2	+	6
Streptomyces lividans TK24	+	Actinomycetales	9	2	+	7
Streptomyces clavuligerus ATCC 27064	+	Actinomycetales	13	3	+	6
Streptomyces viridochromogenes DSM 40736	+	Actinomycetales	4	1	+	5
Kribbella flavida DSM 17836	+	Actinomycetales	7	2	+	1
Conexibacter woesei DSM 14684	+	Solirubrobacterales	1	0	-	0
Micromonospora aurantiaca ATCC 27029	+	Actinomycetales	4	0	+	5
Catenulispora acidiphila DSM 44928	+	Actinomycetales	10	2	+	1
Chitinophaga pinensis DSM 2588	-	Sphingobacteriales	6	1	-	0
Flavobacterium johnsoniae UW101	-	Flavobacteriia	4	0	-	0
Ktedonobacter racemifer DSM 44963	+	Ktedonobacterales	5	0	+	0
Bacillus mycoides DSM 2048	+	Bacillales	2	0	-	3
Collimonas fungivorans Ter331	-	Burkholderiales	1	1	-	1

Chitin resources and bacterial growth

We used four different chitin sources: *N*-acetylglucosamine (chitin monomer), colloidal chitin, small particles of crystal chitin (<0.5 mm) and big particles of crystal chitin (> 2 mm). Purified crystal chitin (poly-N-acetyl-1, 4-β- D-glucosamine, Sigma-Aldrich) was manufactured from shrimp shells. For use in this experiment it was sieved through 2 (big size) or 0.5 (small size) mm mesh filters. Colloidal chitin was prepared from crystal chitin according to Hsu & Lockwood (1975). Their procedure includes the dissolution of bleached chitin in concentrated HCl, suspending the dissolved chitin by adding water, and removing the HCl in several washing steps until the pH of the suspension was between 2.5 to 3.0.

The chitin containing liquid growth medium was prepared by mixing either 0.1 g colloid chitin, 0.5 g *N*-acetylglucosamine, 1 g big (>2.0 mm) or small (< 0.5 mm) size chitin powder in 1 L medium containing 40 mg $MgSO_4 \cdot 7\ H_2O$, 20 mg $CaCl_2 \cdot 2\ H_2O$, 1 g KH_2PO_4. The chitin sources were the only carbon- and energy substrates for bacterial growth. The final pH of media was adjusted to 6.5. The medium was sterilized by heating at 121 ^{0}C for 15 minutes.

Bacterial inocula were prepared by transferring bacterial biomass from agar plates to 1 ml sterilized phosphate buffer to make suspensions with an OD of 0.01 (approximately 10^7 cells/ml), 0.2 ml of these bacterial suspensions was transferred to 100 ml of liquid media with different chitin resources. After inoculation, flasks were put on a shaker (100 rpm) and incubated at 20 ^{o}C. Every two days 1.5 ml samples were taken from the flask and centrifuged, after which the supernatant was stored at – 20 ^{o}C. We took samples for a total period of 24 days.

Chitin degradation was quantified by measuring the amount of NH_4^+-N released from chitin in liquid media (De Boer et al 1996). NH_4^+-N was determined

using an auto analyzer QUAATRO (SEAL). A control treatment, without N-acetylglucosamine or chitin in the growth medium, was included.

Antifungal activity

Antifungal activity of the selected bacteria was tested by an *in vitro* confrontation assay as shown in Figure 1. Bacteria were grown on water-yeast agar for one week in area B of the plate. Next, a PDA agar block of 1 cm Ø from the growing margin of a fungal colony was placed in area A. As a control fungal strains were transferred to agar without the presence of bacteria in area B. After sealing the plates were incubated at 20°C for 2 weeks.

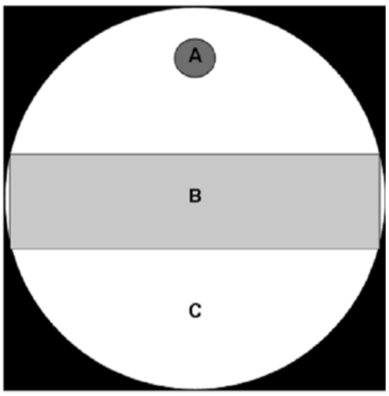

Figure 1 Design of antifungal activity test of bacteria. See text for explanation of the letters

Antifungal activity of bacteria was determined by comparing the fungal mycelium development in area C of control plates with that of bacteria-containing plates. The fungal strains used in the test were: *Mucor hiemalis* (zygomycete) isolated from Dutch coastal dunes, *Apergillus niger* (ascomycete) obtained from the Centraalbureau voor Schimmelcultures (CBS, Utrecht, The Netherlands), and *Rhizoctonia solani* (basidiomycete) obtained from the Instituut voor Rationele Suikerproductie (IRS), Bergen op Zoom, The Netherlands.

Data analysis

The results were statistically examined using Microsoft Excel 2010.

Correlation between chitin degradation rates or antifungal activity and chitinase numbers, chitin binding protein numbers of the bacteria was calculated in PAST (Hammer et al 2001). Antifungal activity was classified in 5 different levels, namely 1 to 5 (see Table 5). These classification levels were used to calculate correlations with other parameters. Hyphal-forming morphology bacteria was categorized by 1(presence of hyphae) and 0 (no hyphal formation). Effects of hyphal morphology were tested for significance using the non-parametric Kruskall-Wallis test in PAST.

Results

Bacterial chitin degradation in liquid media

The release of NH_4^+ from the different chitin resources was determined at 12 time points, every other day of the total incubation period. In Table 2 NH_4^+-N concentration are given at day 1, 12 and 24. For colloidal chitin and crystal chitin we calculated the NH_4^+-N production for two periods namely the accumulation of NH_4^+-N from day 1 to day 12, and from day 12 to day 24. For both periods, we calculated the release rate of NH_4^+-N on a daily basis. Several bacteria showed an increase of NH_4^+-N production during the first 24 h followed by a slow accumulation during the next 11 or 23 days. We attributed this initial quick release to decomposition of easily accessible chitin on the outside of the particles or of soluble chitin oligomers present in the sieved chitin fractions. Therefore, we used the NH_4^+-N release rate during the subsequent period (day 1 – day 12 and day 12 – day 24) as proxy for chitin degrading rate of these bacteria. Since rapid decomposition is to be expected for *N*-acetylglucosamine the NH_4^+-N production during the first 24 h was included in the calculations. The highest rates of substrate degradation are shown in Table 3.

Table 2 NH_4^+-N concentrations at day 1, 12 and 24 after start of the incubations

	N-acetylglucosamine			Colloidal chitin			Small size crystal chitin			Big size crystal chitin		
	Day 1	Day 12	Day 24	Day 1	Day 12	Day 24	Day 1	Day 12	Day 24	Day 1	Day 12	Day 24
Control	0,163	-	-	0	-	-	0	-	-	0	-	-
Streptomyces coelicolor A3 (2)	0,148	0,641	1,394	0,026	0,303	0,801	0,045	0,568	2,492	0,041	0,281	1,337
Streptomyces lividans TK24	0,178	0,282	0,57	0,047	0,127	0,492	0,033	0,077	0,601	0,03	0,073	0,467
Streptomyces clavuligerus ATCC 27064	0,885	1,122	1,419	0,043	0,149	0,292	0,38	0,898	2,083	0,184	0,313	0,932
Streptomyces viridochromogenes DSM 40736	0,941	1,7	2,853	0,058	0,376	0,942	0,337	0,914	2,149	0,163	0,327	0,659
Kribbella flavida DSM 17836	0,272	0,742	0,985	0,036	0,192	0,306	0,029	0,245	0,859	0,033	0,178	0,263
Conexibacter woesei DSM 14684	1,262	1,326	1,36	0,031	0,049	0,228	0,19	0,373	0,682	0,139	0,199	0,256
Micromonospora aurantiaca ATCC 27029	0,839	0,909	0,909	0,04	0,107	0,254	0,282	0,486	1,45	0,159	0,234	0,477
Catenulispora acidiphila DSM 44928	0,157	0,335	0,624	0,022	0,082	0,263	0	0,071	0,128	0	0,052	0,077
Chitinophaga pinensis DSM 2588	1,493	1,546	1,571	0	0,039	0,034	0,222	0,223	0,417	0	0,291	0,552
Flavobacterium johnsoniae UW101	0,748	2,205	2,685	0,056	0,717	2,353	0,168	0,291	0,722	0,093	0,296	0,682
Ktedonobacter racemifer DSM 44963	-	0,903	1,172	0	0,033	0,102	0,332	0,368	0,477	0,12	0,147	0,26
Bacillus mycoides DSM 2048	0,159	1,948	2,71	0	0,044	0,05	0,112	0,202	0,246	0,082	0,118	0,376
Collimonas fungivorans Ter331	1,815	2,842	2,876	0,047	0,602	1,301	0,39	0,424	0,541	0,218	0,252	0,759

Table 3 Release rate of NH_4^+-N from chitinous substrates in liquid media. Data represent the amount of NH_4^+-N (mmol/l) released per day over the periods day 1 – day 12 or day 12 – day 24 (highest rate of the two periods is presented). For N (*N*-acetylglucosamine) substrate, data represent the highest rate for the periods of day 0- day 1, day 1- day 12 and day 12- day 24

	N	C	S	B
Streptomyces coelicolor A3 (2)	0,063	0,042	0,160	0,088
Streptomyces lividans TK24	0,024	0,030	0,044	0,033
Streptomyces clavuligerus ATCC 27064	0,722	0,012	0,099	0,052
Streptomyces viridochromogenes DSM 40736	0,778	0,047	0,103	0,028
Kribbella flavida DSM 17836	0,109	0,014	0,051	0,013
Conexibacter woesei DSM 14684	1,099	0,015	0,026	0,005
Micromonospora aurantiaca ATCC 27029	0,676	0,012	0,080	0,020
Catenulispora acidiphila DSM 44928	0,024	0,015	0,006	0,005
Chitinophaga pinensis DSM 2588	1,330	0,004	0,016	0,026
Flavobacterium johnsoniae UW101	0,585	0,136	0,036	0,032
Ktedonobacter racemifer DSM 44963	ND	0,006	0,009	0,009
Bacillus mycoides DSM 2048	0,163	0,004	0,008	0,022
Collimonas fungivorans Ter331	1,652	0,058	0,010	0,042

N: *N*-acetylglucosamine; B: big size crystal chitin; S: small size crystal chitin; C: colloidal chitin

For most of the bacterial strains the degrading rates of *N*-acetylglucosamine were much higher than for the other substrates (Table 3). The highest rate of *N*-acetylglucosamine degradation was seen for *Collimonas fungivorans* Ter331 followed by *Chitinophaga pinensis* DSM 2588. For degradation of colloidal chitin, the highest degrading rates were observed for *Flavobacterium johnsoniae* UW101 followed by *Collimonas fungivorans* Ter331. Highest rates of

degradation of crystal chitin were observed for Streptomyces *spp.* Degardation rates of small and big crystal chitin were correleted (Table 4).

Table 4 Pearson correlation coefficients for degradation rates of different substrates. Significant correlations (P < 0.05) are indicated in bold

		Degrading rate			
		N	C	S	B
Degrading rate	*N*	-	0.18	-0.17	0.016
	C	-	-	0.10	0.29
	S	-	-	-	**0.74**

N: *N*-acetylglucosamine; B: big size crystal chitin; S: small size crystal chitin; C: colloidal chitin

Antifungal activities

The scores for antifungal activities of the chitinolytic bacterial strains are given in Table 5. The results indicated that bacterial strains showed different antifungal activities in the *in vitro* assay. Overall, most of the *Streptomyces s*trains showed a broader and stronger antifungal activity than other bacteria. *Flavobacterium johnsomiae* UW 101, *Ktedonobacter racefmifer* DSM 44963, *Bacillus mycoides* DSM 2048 and *Collimonas fungivoras* Ter331 showed also strong antifungal activity against one or two fungi.

Two strains, *Conexibacteri woesei* and *Chitinophage pinesis* did not grow well on the media used here. This is most likely the reason why there was no antifungal activity observed for these strains.

Table 5 Classification of *in vitro* antifungal activities of the selected chitinolytic bacteria

Strains	M	A	R	Extra information
Streptomyces coelicolor A3 (2)	5	5	2	
Streptomyces lividans TK24	5	5	4	
Streptomyces clavuligerus ATCC 27064	2	5	5	
Streptomyces viridochromogenes DSM 40736	4	2	3	
Kribbella flavida DSM 17836	1	5	3	
Conexibacter woesei DSM 14684	1	1	1	Did not grow well
Micromonospora aurantiaca ATCC 27029	1	1	2	
Catenulispora acidiphila DSM 44928	1	1	1	
Chitinophaga pinensis DSM 2588	1	1	1	Did not grow well
Flavobacterium johnsoniae UW101	2	3	3	
Ktedonobacter racemifer DSM 44963	1	5	2	
Bacillus mycoides DSM 2048	4	2	2	
Collimonas fungivorans Ter331	2	1	5	

M: *Mucor hiemalis*; A: *Aspergillus niger*; R: *Rhizoctonia solani*. (Classification of antifungal activity: 1: no inhibition; 2: weak inhibition at only one time point; 3: weak inhibition at both time points; 4: weak inhibition at one time point and strong inhibition at another time point; 5: strong inhibition at both time points)

Correlation analysis

The degradation rates of crystal chitin particles, both big and small size were significantly positive related with the number of genes encoding chitin binding proteins but not with the number of chitinase genes. Except this, there was no significant correlation between other parameters and degrading rates of different substrates (Table 6).

The number of chitin-binding proteins in bacteria was also significantly positive correlated with antifungal activity against *Mucor hiemalis*. Numbers of GH18 chitinases were significantly positively correlated with the bacteria's antifungal activity against *Aspergillus niger* (Table 6).

Table 6 Pearson correlation coefficients for the relationship between degradation rates and in vitro antifungal activities with number of chitinases and chitin-binding proteins. Significant correlations (P < 0.05) are indicated in bold

	Degrading rate				Antifungal activity		
	N	C	S	B	*M*	*A*	*R*
GH 18 chitinases numbers	-0.46	-0.21	0.37	0.30	0.10	**0.56**	0.21
GH 19 chitinases numbers	-0.22	-0.17	0.42	0.48	0.24	0.48	0.43
Chitin binding protein numbers	-0.29	-0.11	**0.72**	**0.58**	**0.71**	0.38	0.41

N: *N*-acetylglucosamine; B: big size crystal chitin; S: small size crystal chitin; C: colloidal chitin; M: *Mucor Hiemali*; A: *Aspergillus niger*; R: *Rhizoctonia solani*

Table 7 Non-parametric Kruskall Wallis test Effects of growth morphology (hyphal growth versus unicellular growth) on degradation rates and *in vitro* antifungal activity (Non-parametric Kruskall Wallis test). Given values are Bonferroni corrected P-values

	Degrading rate				Antifungal activity		
	N	C	S	B	*M*	*A*	*R*
Hyphal *versus* non-hyphal	**0.04**	0.94	0.11	0.94	0.81	0.07	0.55

N: *N*-acetylglucosamine; B: big size crystal chitin; S: small size crystal chitin; C: colloidal chitin; M: *Mucor Hiemali*; A: *Aspergillus niger*; R: *Rhizoctonia solani*

The impact of hyphal morphology on degradation of the chitin substrates was only significant for *N*-acetylglucosamine, for which filamentous bacteria had a lower degradation rate then non-filamentous ones (Table 7).

Table 8 Pearson correlation coefficients for the relationships between degradation rates and in vitro antifungal activities. Significant correlations (P < 0.05) are indicated in bold

		Degradation rate			
		N	C	S	B
Antifungal activity	*M*	-0.33	0.19	0.49	**0.62**
	A	**-0.60**	0.01	0.41	0.40
	R	0.19	0.32	0.20	0.41

N: *N*-acetylglucosamine; B: big size crystal chitin; S: small size crystal chitin; C: colloidal chitin; M: *Mucor Hiemali*; A: *Aspergillus niger*; R: *Rhizoctonia solani*

The *in vitro* antifungal activity against fungal strain *Mucor hiemalis* was significantly positively correlated with degradation rates of big crystal chitin particles but not with degradation rates of small size crystal chitin particles or colloidal chitin. Antifungal activity of chitinolytic bacteria against *Aspergillus niger* was significantly negatively correlated with their degrading rates of *N*-acetylglucosamine (Table 8).

Discussion

Bacteria play an important role in the degradation of chitinous resources via hydrolysis of chitin polymers by chitinases (Hoell et al 2010). The chitinolytic system (number of chitinases, modular composition, additional proteins) differs considerably among bacteria and these differences may point at differences in ecological functioning (Bai et al 2015). Next to degradation of chitin, chitinases can be part of an antagonistic system involved in competition with fungi or in defense against bacteriolytic fungi. This antagonistic activity is based on the fact that chitinases can contribute to destabilization of the fungal exterior (cell wall and cell membrane) as chitin is an important structural component of the fungal cell wall (Bowman & Free 2006). In this study we tested, via correlation analysis, whether there is a relationship between the assembly of the chitinolytic system and chitin-degrading abilities or antifungal activities for a set of bacteria.

The results showed that the abilities to degrade the different chitin resources and the chitin monomer *N*-acetylglucosamine differed strongly among the bacterial strains. The positive correlation between degradation of small and big crystal chitin indicates that bacteria use similar mechanisms to degrade these substrates. In particular, strains from the genus *Streptomyces,* showed high decomposition activity with respect to both big and small chitin particles. It has been suggested that the formation of hyphae by these bacteria is important for the degradation of crystal chitin as it allows for penetration of chitin layers (Gooday 1994). Yet, several other hyphal *Actinomycetes* (such as *Kribbella flavida* DSM 17836 and *Catenulispora acidiphila* DSM 44928), showed slow degradation

of chitin particles, in particular of big chitin particles. Moreover, no statistically significant differences were observed in the degradation of chitin particles between hyphal and non-hyphal bacteria. This indicates that the ability to degrade crystal chitin is not necessarily strong for or limited to filamentous bacteria. The significant lower degradation of *N*-acetylglucosamine by hyphal bacteria is not surprising as the unicellular growth form is superior for compounds dissolved in aqueous media (De Boer et al 2005).

The production of multiple chitinases is thought to enable synergistic actions during chitin degradation (Horn et al 2006, Techkarnjanaruk & Goodman 1999). Although most of tested strains with higher numbers of chitinases, such as *Streptomyces* showed strong chitin degradation ability, strain *Micromonospora aurantia* DSM 43813 which produce 12 chitinases was a poor degrader of big size crystal chitin. Chitin binding proteins are also known as lytic polysaccharide monooxygenases (LPMOs). They belong to CAZy as Auxilliary Activity family 10 (Horn et al 2012, Levasseur et al 2013). These proteins were reported to introduce molecular oxygen, and then break the polysaccharide chains without the need of first "extracting" these chains from their crystalline matrix, which promotes further degradation by chitinases (Horn et al 2012, Vaaje-Kolstad et al 2010). In addition, chitin binding proteins of the chitinolytic system of *Streptomyces* have been reported to have direct contributions to chitin degradation (Hoell et al 2010, Schrempf 2001). Our study supports the importance of the role of chitin-binding proteins in crystal chitin degradation and antifungal activity.

Neither the number of chitin-binding proteins nor the number of chitinases was correlated with degradation of colloidal chitin. This shows that the structural property of this chitin substrate that is obtained after dissolution in strong acid and re-precipitation in water is different from that of crystal chitin. Indeed, the fungus *Aphanocladium album* was shown to excrete different chitinases when grown on crystal or colloidal chitin (Studer et al 1992). Interestingly, two non-filamentous bacteria with gliding motilities (*Flavobacterium*

johnsoniae UW101 and *Collimonas fungivorans* Ter331) were the fastest degraders of colloidal chitin. This indicates that motility may be an important property for unicellular bacteria to degrade the (very small) particles in colloidal chitin suspensions.

The *in vitro* inhibition test revealed different responses by the fungi to their confrontation with chitinolytic bacteria. Yet some patterns could be recognized. *Streptomycete* isolates showed a strong inhibition against all fungi. *Streptomycetes* are known as degraders of cellulose and other polymers in soil organic matter (Mccarthy & Williams 1992). They share this niche with fungi, and therefore the production of antifungal compounds may be part of their arsenal to compete with fungi (De Boer et al 2005). Based on the current results it is hard to assess the role of chitinases in this interference competition.

In summary, our study gave additional support for the importance of both the chitinolytic system and other abilities (gliding, hyphal growth) for the ability of bacteria to degrade different chitin resources. With respect to the chitinolytic system, our study lends support to the suggested prominent role for chitin-binding proteins in degradation of crystal chitin. No clear relationships were observed between chitin-degrading abilities and inhibition of fungi, underlining the ideas that a combination of factors is in involved in antifungal activity. Despite the fact that we realize that the incubation conditions used in this study (liquid, 20 ^{o}C) may not be optimal for all the strains, we feel that it is justified to draw the aforementioned general conclusions.

Chapter 4

Composition of chitinolytic soil bacterial community colonizing different chitin resources

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Abstract

Natural resources of chitin consist mainly of exoskeletons of arthropods and fungal cell walls. The amounts of chitin as well as the composition of other compounds co-occurring with chitin polymers differ in these resources. This may require specific abilities of microbes to assess and degrade chitin in a particular resource. In the current study, we examined whether the composition of chitin-containing resources was affecting the assembly of chitinolytic bacterial communities. To this end litterbags containing sterile sand amended with different chitin resources were buried in two natural soils (forest and ex-arable grassland) and colonization by chitinolytic bacteria was examined. Chitin resources included crystalline chitin purified from shrimp shells, fungal (*Aspergillus niger, Mucor hiemalis)* cell walls and cuticles from mealworms. Pyrosequencing of ribosomal gene fragments as well as of the *chiA* catalytic domain were used to analyze the community of bacterial colonizers and the fraction of *chiA*- possessing species therein. The composition of the *chiA* gene harboring bacterial community was mainly affected by soil origin and harvest time, whereas the chitin resources had a minor albeit significant effect. *ChiA* sequences assigned to Beta- and Gamma- *Proteobacteria* were among the dominant enriched bacteria for both forest and grassland soils. Relative abundance of C*hiA* sequences assigned to *Actinobacteria* varied among treatments but was in general much lower than expected on basis of the reported chitinolytic abilities of this phylum. *Firmicutes*-like *ChiA* sequences increased during prolonged incubation. The composition of chitinolytic bacterial isolates that were obtained from the chitin-enriched sand patches was only partly in agreement with the directly retrieved *ChiA* sequences. Most notably was the lack of *ChiA* sequences assigned to Bacteroidetes whereas several chitinolytic strains (*Flavobacterium*, *Chitinophaga*) belonging to this phylum were isolated Overall, the results point at soil origin (composition of community of indigenous soil chitinolytic bacteria) as

the most important factor steering the composition of chitinolytic bacterial taxa colonizing different chitin resources.

1. Introduction

Soil bacteria play a key role in the decomposition of natural polymers, such as cellulose and chitin. Chitin is the second most abundant natural polymer after cellulose (Gooday, 1990) and its annual production ranges from 2.8×10^7 Mg yr^{-1} for freshwater ecosystems to 1.3×10^9 Mg yr^{-1} for marine ecosystems, mainly consisting of chitin in exoskeletons of arthropods (Cauchie, 2002). In terrestrial habitats, fungal propagules (hyphae, spores etc.) or remainders of fungi (Smrz and Catska, 2010) are the major natural resources of chitin. However, until now there is no reliable estimate of the annual chitin production in terrestrial ecosystems. Microbial chitin degradation involves specialized enzymes, called chitinases, which are widespread among bacteria. In soil, most abundant chitinolytic bacteria belong to the phyla *Actinobacteria, Firmicutes,* and *Proteobacteria* (Hjort et al., 2010; Golinska and Dahm, 2011).

The effect of chitin addition on soil microbial communities has been investigated in several studies (Krsek and Wellington, 2001; Sato et al., 2010; Cretoiu et al., 2013; Kielak et al., 2013) In most of these studies the resources of chitin that were added to soil consisted of (partly) purified crystalline chitin or milled material of exoskeletons of sea arthropods, such as shrimps or crabs. However, in terrestrial ecosystems chitin is mainly present in fungal cell walls. The structure of chitin in fungal cell walls and in cuticles of arthropods is similar, *i.e.* the so-called alpha-chitin structure (Jeuniaux, 1982). The chitin content of both arthropod cuticles and fungal cell walls can vary strongly, with reported ranges of 10 – 40 % (Ebner, 2002; Wu et al., 2005; Finke, 2007).

However, a major difference between arthropod and fungal chitin resources is the composition of the co-occurring structural compounds and proteins. In fungal cell walls, chitin is embedded in a matrix of glucans, mannans and glycoproteins (Bowman and Free, 2006). Crustacean exoskeletons may have a high content of minerals, such as calcite, and insect exoskeletons also contain

proteins and lipids (Moussian et al., 2005; Raabe et al., 2006). Some fungal cell walls can also contain chitosan, a derivative of chitin formed by partial deacetylation of the chitin (Raafat et al., 2008). In nature, chitosan occurs mostly in the cell walls of *Zygomycetes* (Synowiecki and Al-Khateeb, 2003). In addition to differences in associated compounds, also the ultrastructure of chitin fibres differs between arthropods and fungi (Bowman and Free, 2006; Raabe et al., 2006).

Due to the aforementioned differences between fungal cell walls and arthropod exoskeletons, we expect that chitinolytic bacteria need different properties to get access to chitin in different natural chitin resources. Therefore, we hypothesize that the composition of chitinolytic bacterial communities will be affected by the nature of the added chitin resource. To test this hypothesis we followed the numbers and composition of bacterial colonizers of buried litterbags filled with sterile sand containing different marine and terrestrial chitin resources. The composition of chitinolytic bacterial colonizers of the chitin-containing sand patches was studied for a grassland and forest soil using DNA-based (sequencing of the catalytic domain of *ChiA* genes) and cultivation based (chitin agar) techniques. *ChiA* was selected as this member of the family 18 glycoside hydrolases is widespread among bacteria and encode for endochitinase which is an essential enzyme for the degradation of chitin (Karlsson and Stenlid, 2008; Kharade and McBride, 2014).

2. Material and method

2.1 Chitin resources

Table 1 Chitin resources used and the amounts added to the sand

Chitin resources	Chitin content (%)	Chitin resource addition (g/100g soil)	Reference
Crystalline chitin	>95	0,5	Sigma
Fungal cell wall of *Mucor hiemalis**	11	1	(Ebner, 2002)
Fungal cell wall of *Aspergillus niger*	24	1	(Wu et al., 2005)
Mealworm cuticle	22	1	(Andersen, 2002)

* does also contain chitosan

The chitin resources used in this experiment are shown in Table 1. Crystalline chitin was purified from shrimp exoskeletons (Sigma-Aldrich, St. Louis, USA), and sieved before use (< 4mm). Fungal cell wall material was prepared from *Mucor hiemalis* (*Zygomycetes*, isolated from Dutch coastal dunes) and *Apergillus niger* strain 400 (obtained from the collections of the Fungal Biodiversity Centre, CBS-KNAW, Utrecht, The Netherlands). The fungi were grown in liquid media consisting of mycological peptone (Becton, Dickinson and Company, Pont de Claix, France) 3.0 gL^{-1}, malt extract (Becton, Dickinson and Company, Pont de Claix, France) 17.0 gL^{-1}, and the pH was adjusted to 5.4. Cultures were incubated at 37 °C for 48 h on a rotary shaker operating at low speed (80 rpm). Fungal cell walls were obtained according to the method of Momany et al. (2004): fungal mycelium was collected by filtering the suspension through a #1 Whatman filter and cleaned using a salt solution (0.5 M NaCl). Subsequently, the mycelium was transferred to a disruption buffer (DB) solution (20 mM Tris, 50 mM EDTA, pH 8.0) and fragmented using a cell disruptor Sonifier B-12 (Branson sonic power company, Danburg, Connecticut) until microscopic examination revealed empty hyphae (hyphal ghosts). Cell walls were collected by centrifugation at 3000 *g* for 10 min. The pellet containing the cell wall fraction was washed by stirring in disruption buffer for 4 h at 4 °C followed by a washing procedure with demineralized water at the same conditions. The pellet was dried by vacuum filtration and lyophilized. At the end, dried fungal cell wall material was grinded and sieved (mesh <4 mm).

Cuticles of meal worms (larvae of Darkling Beetle) obtained from the Animal Ecology department of NIOO-KNAW were frozen with liquid nitrogen, grinded and sieved (mesh < 4 mm).

2.2 Experimental design

Sterilized nylon bags (4.8 × 5.9 cm, mesh-size 10×10 µm) were filled with 15 g of purified sea sand (Honeywell Specialty Chemicals Seelze GmbH, Seelze,

Germany). Before the sand was added to nylon bags it had been mixed with one of the 4 chitin resources (Table 1) or no chitin resource (control), then the mixture were autoclaved, after that the moisture of the mixture was adjusted with demineralized water to 10% (w/w). The nylon bags containing sand plus chitin resources were buried in approximately 100 g homogenized soil in Petri dishes. We used two sandy soils, an ex-arable grassland soil (pH_{water} 5.8; organic matter content 4.7% and a mixed forest soil (pH_{water} 3.6; organic matter content 4.8%), both from the Veluwe, an area in the center of the Netherlands with soils developed on glacial sandy deposits. Details on location and soil characteristics for the ex-arable soil are provided by Maly et al. (2000) and for the forest soil by Folman et al. (2008). Samples were collected from the upper 0-10 cm mineral layer of both soils. Soils were homogenized by sieving (< 4mm).

Petri dishes were sealed with parafilm and incubated at 20 °C. There were 4 replicates for each treatment and incubation period. Nylon bags were removed after 3 and 7 weeks of incubation.

2.3 Isolation of chitinolytic bacteria

After harvest, 1 g of sand was taken out of each of the nylon bags and mixed with 9 ml of sterile water. This sand suspension was shaken for 1 hour (200 rpm) and dilution-plated on chitin-water agar (layer of chitin-agar on top of water-agar) in order to enumerate and isolate chitin degrading bacteria. The composition of the media was:

1) Water-agar: 15 g agar per litre water
2) Chitin-agar (500 ml): colloidal chitin, 1 g; Agar, 10 g; MES (2-(N-morpholino) ethanesulfonic acid), 1.0 g; KH_2PO_4, 0.5 g; $MgSO_4.7H_2O$, 0.02 g; $CaCl_2.2H_2O$, 0.01 g; Yeast-extract, 0.05 g. pH was adjusted to 6.5.

Plates were incubated at 20 ^{O}C. After one week of incubation, chitin degrading isolates (indicated by clear haloes around bacterial colonies) were picked and transferred to 0.1 TSB plate for purification (De Boer et al., 1998).

To identify the chitinolytic bacterial isolates, 16S rDNA gene fragments were amplified by colony PCR using primer pair 27F/1492r (Weisburg et al., 1991). Quality and purity of PCR products were checked by Nanodrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, Wilmington, USA) as well as by gel electrophoresis. Amplicons were sequenced by the Sanger method (Macrogen, Seoul, Korea). 16S rDNA sequences were blasted against sequences available in the Ribosomal Database Project (RDP: http://rdp.cme.msu.edu). A sequence similarity of 97% was applied as the threshold for assigning a sequence to a known bacterial genus.

2.4 Total community DNA isolation

0. 5 g sand was taken out of each of the nylon bags and total DNA was extracted using the PowerSoil kit (MoBio Laboratories, Inc., Carlsbad, CA, USA) according to the company guidelines. DNA purity and quality was checked using Nanodrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, Wilmington, USA) as well as by agarose gel electrophoresis.

2.5 Quantification of 16S rDNA copies of bacterial community

Collimonas fungivorans Ter331 was used as template for preparation of clones containing 16S rDNA genes using a kit of the pGEM-T Vector system (Promega, Madison, USA). The plasmid of the obtained clones was isolated using the PowerSoil kit (MoBio Laboratories, Inc., Carlsbad, USA). After that, a standard series of 10^1 to 10^8 copy numbers of 16S rDNA gene fragments per reaction was made based on the plasmid concentration measured by Nanodrop 2000 UV-Vis Spectrophotometer. Quantitative PCR of samples was performed on Rotor-gene 3000 (Westburg, Leusden, the Netherlands) using Qiagen SYBR green mix. Primers used were Eub338/Eub518 (Fierer et al., 2005), The copies number of the 16S rDNA gene of each sample was calculated using a standard curve.

2.6 Pyrosequencing analysis of bacterial 16S rDNA gene

Barcoded pyrosequencing of the V4 region of the small subunit of the bacterial ribosomal gene was carried out as previously described (Vos et al., 2012). The forward primer consisted of primer A from 454 Life Sciences followed by a 10 base sample specific barcode, the 2-base linker sequence GT and the conserved bacterial primer sequence 515F. The reverse primer consisted of 454 Life Sciences primer B, a 10 base long sample specific barcode, the 2-base linker sequence GG and the conserved bacterial primer 806R. Amplicon products were checked by gel electrophoresis, cleaned, pooled according to the concentration measured by Nanodrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, Wilmington, USA) . The products were sequenced on a Roche 454 GS FLX system (Macrogen, Seoul, Korea).

Reads were analyzed using a Snakemake workflow (Koster and Rahmann, 2012) that follows the SOP for 454 data in Mothur, version 1.32.1 (Schloss et al., 2009). The flowgrams were demultiplexed with a mismatch of 2 to the barcode, 3 mismatches to the primer and trimmed to 430 flows. Flowgrams were corrected (remove sequencing noise) using the shhh.flows command, which is the Mothur implementation of the original PyroNoise algorithm (Quince et al., 2011).

The merged sequences from above steps were aligned to the bacterial reference alignment provided on the Mothur website (http://www.mothur.org/wiki/Silva_reference_alignment) which is SILVA 102 release of the SSURef database (Quast et al., 2013). In order to reduce more sequence errors, the pre.cluster command was performed to merge sequences within 2 mismatches. After that, the chimera.uchime command was used to remove chimeric sequences (Edgar et al., 2011).

OTUs were formed using the dist.seqs command and average neighbor clustering at maximum distance of 0.03. All sequences were taxonomically classified using the Mothur implementation of the RDP classifier (Wang et al., 2007) and using the training set (version 9) provided on the Mothur website (http://www.mothur.org/wiki/RDP_reference_files). A consensus taxonomy of

each OTU was determined using the classify.otu command.

Representative sequences for each OTU were re-aligned to the Silva reference alignment and a neighbor joining tree was created using the clearcut program (Sheneman et al., 2006). Taxonomic classification and OTU clustering data were combined into the BIOM format (McDonald et al., 2012). Then the BIOM files were fed to the Mothur platform for normalization by the minimum reads of samples (525 reads). Normalized BIOM files were fed to Megan 5 (Huson et al., 2007) for further downstream statistical analysis to analyze the community composition. Richness (based on numbers of different OTUs) and diversity (based on Shannon index) of the bacterial community was calculated in Mothur based on normalized files.

2.7 Pyrosequencing of catalytic domain of bacterial *chiA* gene

The bacterial *chiA* gene catalytic domain was amplified as previously described by Kielak et al. (2013). The forward primer consisted of primer A from 454 Life Sciences followed by a 10 base sample specific barcode and the conserved bacterial *chiA* primer sequence GA1F (Krsek and Wellington, 2001). The reverse primer consisted of 454 Life Sciences primer B, a 10 base long sample specific barcode and the conserved bacterial *chiA* primer GA1R (Williamson et al., 2000). Then amplifications of all samples were produced on a Peltier 96-well thermal cycler (BIOzym-Landgraaf, the Netherlands). The thermal cycling conditions were as follows: initial denaturation step (95°C for 5 min), 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and elongation at 72°C for 1 min, final elongation step for 10 min at 72°C. Amplicon products were checked by gel electrophoresis, cleaned and pooled according to the concentration measured by Nanodrop 2000 UV-Vis Spectrophotometer (Thermo scientific, USA). Then the product was sequenced on a Roche 454 GS FLX system (Macrogen, Seoul, Korea).

Sequence reads were analyzed in Mothur (Schloss et al., 2009). First, quality control of sequences was performed on the basis of the following criteria: mean quality of reads >25, maximum zero ambiguous bases, maximum homopolymers 8. Then the sequences were trimmed to remove primers and barcodes. After that, ChimeraSlayer was used to remove potential chimeras. Remaining reads were then translated into amino acid sequences. Sequences containing internal stop codons and unidentified amino acids due to sequencing errors were removed. Each translated sequence was used as a Blast-P query against a 2,171 sequence database obtained from CAZy (Cantarel et al., 2009) using a 10^{-20} E value cutoff. Qualified amino acid sequences were aligned using MAFFT (version 7) (Katoh and Standley, 2013), together with the corresponding region of the reference sequences from CAZy. Distance matrices of aligned sequences were calculated by the protdist program of PHYLIP 3.67 package (Felsenstein, 2005). The obtained matrix was applied to Mothur for complete linkage clustering. Sequences were then assigned to operational taxonomic units (OTUs) based on a 7 % dissimilarity cutoff. This cutoff was used based on slope stabilization by plotting the number of unique (OTUs) at different OTU cut-off values. After that, obtained sequences were normalized in the Mothur platform by the minimum reads of samples (498 reads). These data were used to assess richness and diversity of the *chiA* gene harboring bacterial community. Finally, to determine the chitinolytic community composition, *chiA* genes affiliated with different bacterial species were identified based on BLAST-P analysis (threshold: e value of 10^{-20}) at NCBI (National Center for Biotechnology Information website, http://www.ncbi.nlm.nih.gov).

2.8 Data analysis

Data were analyzed by SPSS (version 19.0). Normality of data was checked with the Shapiro–Wilk's test. An appropriate transformation was applied (log transformation) for the data which failed the test. Homogeneity of variances was assessed by Levene's test. Tukey's HSD post hoc test of One-way ANOVA was

applied to test differences in relative abundance depending on the factors soil origin, harvest time and chitin resources (a value of $P< 0.05$ was considered significant). Tamhane's T2 post hoc test was applied when equal variance was not assumed.

All ordination analyses were performed in Canoco version 4.5 (Šmilauer and Lepš, 2014). Canonical Correspondence Analysis (CCA) was used to test if the composition of the enriched bacterial community and the *chiA*- possessing species were related to soil origin, chitin resources or harvest time. Significance of canonical axes was assessed by the forward approach using Monte Carlo permutation tests under the reduced model.

3. Results

3.1 Q-PCR of 16S rDNA genes

For both soils and at both incubation periods, DNA could be extracted from sand in nylon bags containing chitin resources but not from the bare sand controls. The number of bacterial 16S rDNA gene fragments in sand in nylon bags was in the range of 10^6 to 10^7 and was not significantly different between treatments (chitin resources and soil origin) (Figure 1). After 7 weeks of incubation, the numbers of 16S rDNA genes were still in the same range.

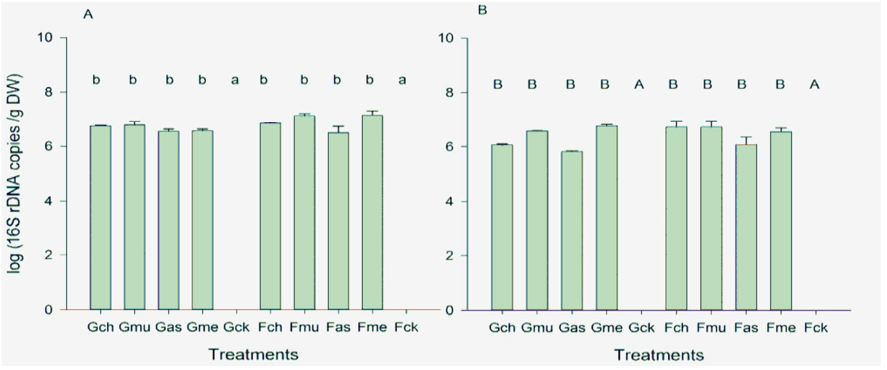

Figure 1 Quantification of 16S rDNA gene fragments in buried nylon bags containing pure sand amended with different chitin resources (ch: crystal chitin; mu: *Mucor hiemalis* cell wall; as:

Aspergillus niger cell wall; me: mealworm cuticle; ck: control) at A: first harvest (3 weeks) ; B: second harvest (7 weeks) (F: forest soil; G: grassland soil). Different letters above treatments indicate significant differences ($P < 0.05$)

3.2 Pyrosequencing of bacterial 16S rDNA genes

Table 2 Observed richness estimates and Shannon diversity estimates of bacterial community. (CHI: crystalline chitin; MUC: *Mucor hiemalis* cell wall; ASP: *Aspergillus niger* cell wall; MEA: mealworm cuticle). Different letters indicate significant differences for the 4 chitin substrate treatments per soil and per harvest time.

			Average score ± Standard Error			
			Richness estimator		Diversity index	
			Sobs[a]	Mark	Shannon	Mark
3 weeks	Grassland soil	CHI	33±8	A	1,99±0,31	B
		MUC	38±8	A	1,90±0,28	B
		ASP	34±10	A	1,73±0,27	A
		MEA	67±2	B	2,97±0,21	C
	Forest soil	CHI	66±6	b	2,95±0,17	B
		MUC	69±5	b	2,99±0,23	B
		ASP	56±4	a	2,60±0,19	A
		MEA	86±4	c	3,37±0,08	C
7 weeks	Grassland soil	CHI	38±1	B	2,05±0,12	B
		MUC	42±9	B	2,11±0,41	B
		ASP	26±9	A	1,28±0,50	A
		MEA	51±5	C	2,46±0,32	C
	Forest soil	CHI	36±6	a	2,02±0,30	A
		MUC	65±6	c	2,84±0,05	C
		ASP	41±5	ab	1,83±0,15	A
		MEA	46±5	b	2,61±0,17	B

[a]Observed richness (numbers of bacterial OTUs) based on average of 4 replicates of each treatment.

Pyrosequencing of 16S rDNA gene fragments extracted from sand in nylon bags was successful for all treatments except the bare sand controls. The original sequences were submitted to European Nucleotide Achieve (ENA) database under accession number PRJEB9706. After quality check of the sequences, on average

1479 reads per replicate were left. The observed richness and diversity of the bacterial community is shown in Table 2. Richness and diversity of the bacterial communities were highest for the mealworm cuticle treatment in both soils at the first harvest and also in the grassland soil at the second harvest. Sand containing *Aspergillus niger* cell walls had the lowest diversity.

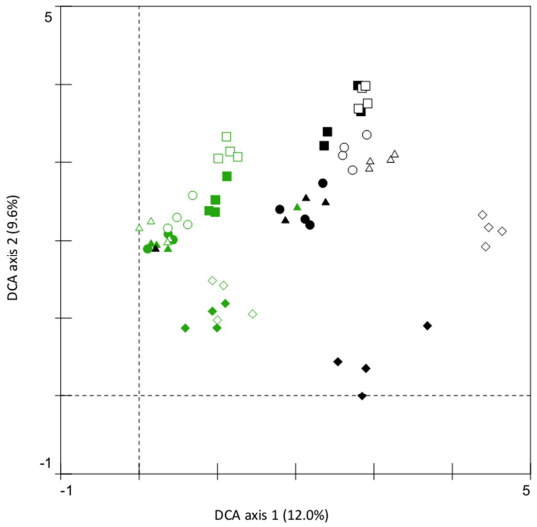

Figure 2 Detrended correspondence analysis (DCA) based on OTUs of total bacteria. Green symbols: grassland soil; black symbols: forest soil. Circle: crystal chitin; triangle: *Mucor hiemalis* fungal cell wall; diamond: *Aspergillus niger* cell wall; square: mealworm cuticle. Closed symbols: 3 weeks after inoculation; open symbols: 7 weeks after inoculation.

DCA analysis showed that soil bacterial communities colonizing sand patches were clustered by soil, harvest time and chitin resources (Figure 2), and forward selection in CCA showed that all of these factors were significantly influencing total bacterial community composition ($p < 0.01$).

Table 3 Relative abundances (%) of bacterial classes (based on pyrosequencing of 16S rDNA fragments) in buried nylon bags containing pure sand amended with different chitinous resources and buried in grassland soil. Given is the average relative abundance from four replicates calculated as the ratio between class abundance and total number of sequences in the treatment. Standard deviation is given in brackets. Sequences represented by less than 1% of the total sequences are grouped and presented as "Others". (CHI: crystal chitin; MUC: Mucor hiemalis cell wall; ASP: Aspergillus niger cell wall; MEA: mealworm cuticle). Differences between treatments were given by superscript letter on the numbers.

	Harvest 1 (t = 3 weeks)				Harvest 2(t = 7 weeks)			
Bacterial Classes	CHI	MUC	ASP	MEA	CHI	MUC	ASP	MEA
Actinobacteria	2,4^{ab} (0,7)	0,1^{a} (0,1)	7,3^{c} (3,6)	4,4^{bc} (1,6)	12,8^{d} (0,4)	0,7^{a} (0,4)	4,9^{bc} (1,2)	3,2^{ab} (0,2)
Bacteroidia	37,8^{a} (10,5)	29,2^{a} (9,8)	6,2^{b} (0,6)	7,3^{b} (3,8)	12,2^{b} (1,3)	13,8^{b} (2,8)	4,0^{b} (0,9)	3,9^{b} (2,2)
Acidobacteriia	0,1^{a} (0,2)	1,6^{c} (0,1)	0,7^{b} (0,1)	0,1^{a} (0,0)	0,2^{a} (0,2)	0,4^{ab} (0,2)	< 0.1	< 0.1
Bacilli	< 0.1	< 0.1	< 0.1	9,6^{b} (1,3)	0,1^{a} (0,0)	< 0.1	< 0.1	16,7^{c} (3,0)
Phycisphaerae	< 0.1	< 0.1	< 0.1	0,1^{a} (0,0)	2,8^{b} (0,0)	2,7^{b} (0,6)	< 0.1	3,5^{b} (1,1)
Alphaproteobacteria	14,4^{ab} (3,7)	6,8^{a} (3,5)	4,5^{a} (3,5)	14,5^{ab} (1,5)	44,3^{c} (4,1)	39,2^{c} (5,4)	19,0^{b} (6,3)	23,3^{b} (5,9)
Betaproteobacteria	9,4^{c} (0,4)	< 0.1	0,3^{a} (0,0)	0,2^{a} (0,2)	1,5^{a} (1,1)	6,4^{b} (1,2)	0,3^{a} (0,1)	1,4^{a} (0,5)
Deltaproteobacteria	0,1^{a} (0,2)	0,4^{a} (0,5)	0,8^{ab} (0,1)	1,0^{b} (0,1)	< 0.1	0,7^{ab} (0,0)	0,6^{ab} (0,5)	1,9^{c} (0,5)
Gammaproteobacteria	34,1^{ab} (4,8)	50,7^{cd} (10,9)	79,9^{f} (5,2)	59,9^{de} (2,1)	24,0^{a} (5,4)	34,2^{ab} (9,2)	69,2^{ef} (3,7)	43,4^{bc} (4,9)
Others	1,8^{a} (1,3)	1,1^{a} (1,2)	0,3^{a} (0,4)	2,7^{a} (3,2)	2,2^{a} (1,0)	2,0^{a} (1,1)	2,0^{a} (0,9)	2,8^{a} (1,1)

As shown in Tables 3 and 4, *Proteobacteria* dominated the bacterial community in the sand patches in grassland soil in all treatments at both harvests. Gamma- and alpha-*Proteobacteria* were the dominant classes with a clear

increase in relative abundance of alpha-*Proteobacteria* at the second harvest. Most of the Gamma-Proteobacteria could be assigned to the families *Pseudomonadaceae* and *Xanthomonodaceae* (Supplementary Table 1). Alpha-proteobacterial sequences were divided over more families but the clear increase at the second harvest was mainly caused by an increase of the families *Sphingomonadaceae, Caulobacteraceae and Rhizobiaceae* (Supplementary Table 1). Bacterial family *Bacteroidaceae* were relatively abundant in the crystalline chitin and *Mucor hiemalis* cell wall treatments.

Table 4 Relative abundances (%) of bacterial classes (based on pyrosequencing of 16S rDNA fragments) in buried nylon bags containing pure sand amended with different chitinous resources and buried in forest soil. Given is the average relative abundance from four replicates calculated as the ratio between class abundance and total number of sequences in the treatment. Standard deviation is given in brackets. Sequences represented by less than 1% of the total sequences are grouped and presented as "Others". (CHI: crystal chitin; MUC: *Mucor hiemalis* cell wall; ASP: *Aspergillus niger* cell wall; MEA: mealworm cuticle). Differences between treatments were given by superscript letter on the numbers.

	Harvest 1 (t = 3 weeks)				Harvest 2(t = 7 weeks)			
Bacterial Classes	CHI	MUC	ASP	MEA	CHI	MUC	ASP	MEA
Actinobacteria	23,0^{b} (2,9)	1,1^{a} (1,1)	1,2^{a} (0,5)	1,1^{a} (0,9)	39,6^{c} (3,5)	0,8^{a} (0,4)	6,8^{a} (4,8)	22,3^{b} (4,8)
Bacteroidia	< 0.1	12,5^{b} (2,2)	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	0,2^{a} (0,0)
Acidobacteriia	1,6^{a} (1,2)	3,7^{a} (1,0)	6,3^{a} (1,3)	2,7^{a} (0,7)	21,4^{bc} (2,7)	25,1^{c} (8,6)	66,3^{d} (12,1)	8,4^{ab} (5,8)
Bacilli	< 0.1	< 0.1	< 0.1	12,0^{b} (4,5)	0,5^{a} (0,2)	< 0.1	0,2^{a} (0,1)	32,9^{c} (7,3)
Gemmatimonadetes	< 0.1	< 0.1	< 0.1	1,8 (0,8)	< 0.1	< 0.1	< 0.1	< 0.1
Phycisphaerae	< 0.1	< 0.1	< 0.1	0,2^{a} (0,1)	< 0.1	< 0.1	0,1^{a} (0,0)	< 0.1
Alphaproteobacteria	14,4^{c} (1,4)	12,2^{bc} (3,2)	1,5^{a} (0,7)	14,3^{c} (7,4)	2,7^{ab} (0,1)	35,6^{d} (9,8)	3,5^{ab} (1,2)	2,0^{ab} (1,5)
Betaproteobacteria	0.00	1,4^{b} (0,1)	0,2^{a} (0,2)	0.00	0,2^{a} (0,2)	0,1^{a} (0,2)	0,1^{a} (0,1)	< 0.1
Deltaproteobacteria	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Gammaproteobacteria	60,7^{b} (9,2)	68,8^{b} (4,3)	90,4^{c} (13,1)	66,2^{b} (4,6)	34,5^{a} (2,2)	36,7^{a} (3,5)	21,4^{a} (1,9)	33,4^{a} (9,1)
Others	0,3^{a} (1,6)	0,3^{a} (0,3)	0,4^{a} (0,4)	1,7^{a} (0,3)	1,0^{a} (0,5)	1,2^{a} (0,9)	1,7^{a} (1,2)	0,9^{a} (0,4)

In forest soil Gamma- *Proteobacteria* was the dominant group that had colonized chitin-containing sand patches at the first harvest, mostly represented by the family *Pseudomonadaceae* (Supplementary Table 1). However, for the sand

enriched with *Aspergillus* cell wall an extremely high amount of *Enterobacteriaceae* was observed. At the second harvest of the forest soil Gamma- *Proteobacteria* were still one of the major classes but now not only *Pseudomonadaceae* but also *Xanthomonodaceae* and *Sinobacteraceae* were strongly represented. A strong contrast with the grassland soil was seen for acidobacteria (class *Acidobacteriia*) which had become one of the dominating groups of colonizers at the second harvest in the forest soil. Actinomycetes (class *Actinobacteria*) were only prominently in the forest soil sand patches containing crystalline chitin (both harvests) or mealworm cuticle (second harvest). Interestingly, both for the forest and grassland soil a high abundance of *Bacilli* was restricted to patches containing mealworm cuticles. *Bacteroidia* were far less abundant in sand patches in forest soil than in grassland soil.

3.3 Culturable chitinolytic bacteria

The identity of the isolated chitinolytic bacteria is shown in Supplementary table 2. At the first harvest, isolates that colonized chitin-containing sand from grassland soil samples were mainly belonging to the genera *Flavobacterium*, *Chitinophaga* and *Pseudomonas.* In the second harvest several isolates were obtained from the genera *Janthinobacterium, Paenibacillus* and *Stenotrophomonas*. Isolates obtained from sand patches that had been buried in the forest soil were mostly belonging to the genera *Paenibacillus, Bacillus*, *Janthinobacterium* and *Collimonas*. No chitinolytic isolates were obtained from the *Aspergillus niger* cell wall treatment of forest soil. Neither of the soils showed a clear indication for selection of cultivable chitinolytic bacteria belonging to a particular genus by one of the chitin resources used.

3.4 Pyrosequencing analysis of catalytic domain of *chiA* gene

Table 5 Observed richness and Shannon diversity estimates based on sequences of *chiA gene* fragments in DNA extracted from buried nylon bags containing pure sand amended with different chitin resources. (CHI: crystalline chitin; MUR: *Mucor hiemalis* cell wall; ASP: *Aspergillus niger* cell wall; MEA: mealworm cuticle). Different letters indicate significant differences for the 4 chitin substrate treatments per soil and per harvest time.

			Average score ± Standard Error			
			Richness estimator		Diversity index	
			Sobs[a]	Mark	Shannon	Mark
3 weeks	Grassland soil	CHI	36±25	AB	1,05±0,42	A
		MUC	18±2	B	0,66±0,14	A
		ASP	14±2	AB	0,91±0,21	A
		MEA	11±1	A	0,79±0,38	A
	Forest soil	CHI	16±5	ab	0,79±0,41	A
		MUC	14±1	a	0,97±0,39	Ab
		ASP	-	-	-	-
		MEA	22±4	b	1,17±0,61	B
7 weeks	Grassland soil	CHI	44±11	B	1,51±0,45	AB
		MUC	39±7	B	1,81±0,42	B
		ASP	-	-	-	-
		MEA	16±7	A	0,76±0,34	A
	Forest soil	CHI	32±5	a	1,80±0,56	A
		MUC	24±7	a	1,40±0,57	A
		ASP	-	-	-	-
		MEA	31±8	a	2,25±0,22	A

[a]Observed richness (number of bacterial OTUs) as based on the average of 4 replicates of each treatment.

On average 1045 reads per replicate were obtained after quality control of sequence data. The original sequences were submitted to European Nucleotide Achieve (ENA) database under accession number PRJEB9708. The observed richness and the diversity of the *chiA* gene harboring bacterial community are shown in Table 5. The relative abundance of bacterial classes as based on *chiA* sequences is shown in Tables 6 and 7.

Table 6 Relative abundances (%) of chitinolytic bacterial classes (based on pyrosequencing of catalytic domain of chiA) in buried nylon bags containing pure sand amended with different chitinous resources and buried in grassland soil. Given is the average relative abundance from four replicates calculated as the ratio between class abundance and total number of sequences in the treatment. Standard deviation is given in brackets. Sequences represented by less than 1% of the total sequences are grouped and presented as "Others". (CHI: crystal chitin; MUC: Mucor hiemalis cell wall; ASP: Aspergillus niger cell wall; MEA: mealworm cuticle). Differences between treatments were given by superscript letter on the numbers.

	Harvest 1 (t = 3 weeks)				Harvest 2(t = 7 weeks)			
	CHI	MUC	ASP	MEA	CHI	MUC	ASP	MEA
Beta-proteobacteria	87,9^{d} (9,1)	73,5^{cd} (13,7)	24,9^{a} (3,3)	63,5^{bc} (9,3)	77,3^{cd} (10,3)	49,7^{b} (9,1)	-	80,7^{cd} (10,8)
Gamma-proteobacteria	1,5^{a} (1,0)	21,3^{bc} (4,8)	59,3^{d} (13,4)	27,8^{c} (1,4)	10,0ab (1,6)	16,5^{abc} (6,7)	-	11,7^{ab} (9,5)
Bacilli	0,1^{a} (0,1)	0,1^{a} (0,0)	< 0.1	1,9^{b} (0,1)	3,8^{c} (1,2)	2,7^{bc} (0,6)	-	2,9^{bc} (0,1)
Actinobacteria	7,6^{a} (5,2)	3,7^{a} (2,0)	15,1^{b} (1,3)	5,1^{a} (0,0)	6,4^{a} (1,0)	24,4^{c} (3,2)	-	2,8^{a} (0,4)
Acidobacteriia	0,1 (0,0)	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	-	0,1 (0,0)
Delta-proteobacteria	< 0.1	0,5^{b} (0,2)	< 0.1	0,1^{a} (0,0)	< 0.1	2,2^{c} (0,1)	-	0,2^{a} (0,1)
Others	2,9^{bc} (0,2)	1,0^{ab} (0,8)	0,7^{a} (0,2)	1,5^{ab} (0,4)	2,5^{b} (0,1)	4,5^{c} (1,8)	-	1,6^{ab} (1,0)

Table 7 Relative abundances (%) of chitinolytic bacterial classes (based on pyrosequencing of catalytic domain of chiA) in buried nylon bags containing pure sand amended with different chitinous resources and buried in forest soil. Given is the average relative abundance from four replicates calculated as the ratio between class abundance and total number of sequences in the treatment. Standard deviation is given in brackets. Sequences represented by less than 1% of the total sequences are grouped and presented as "Others". (CHI: crystal chitin; MUC: Mucor hiemalis cell wall; ASP: Aspergillus niger cell wall; MEA: mealworm cuticle). Differences between treatments were given by superscript letters on the numbers.

	Harvest 1 (t = 3 weeks)				Harvest 2(t = 7 weeks)			
	CHI	MUC	ASP	MEA	CHI	MUC	ASP	MEA
Beta-proteobacteria	17.8^{b} (1.6)	26.4^{a} (4.0)	-	9.1^{d} (2.1)	12.5^{c} (1.3)	2.0^{e} (1.5)	-	8.1^{cd} (3.7)
Gamma-proteobacteria	24,0^{a} (6,4)	70,2^{bc} (10,3)	-	77,6^{c} (12,4)	24,5^{a} (6,0)	59,2^{b} (3,1)	-	20,1^{a} (1,7)
Bacilli	0,7^{a} (0,1)	< 0.1	-	1,7^{a} (0,1)	51,7^{c} (4,3)	13,9^{b} (4,9)	-	20,6^{b} (7,9)
Actinobacteria	57,3^{d} (9,5)	1,3^{a} (0,5)	-	11,1^{b} (0,1)	11,2^{b} (1,6)	23,8^{c} (2,6)	-	50,4^{d} (2,5)
Acidobacteriia	< 0.1	1.3^{b} (0.1)	-	< 0.1	< 0.1	0.2^{a} (0.0)	-	< 0.1
Delta-proteobacteria	< 0.1	< 0.1	-	<0.1	<0.1	< 0.1	-	<0.1
Others	0,2^{a} (0,1)	0,9^{c} (0,0)	-	0,6^{b} (0,0)	<0.1	1,0^{c} (0,0)	-	0,9^{c} (0,0)

The presence of *Aspergillus* cell walls in sand resulted in very low amounts of *chiA* sequences for both soils (Table 5). The other treatments did not show consistent differences in richness and diversity of bacterial *ChiA* genes.

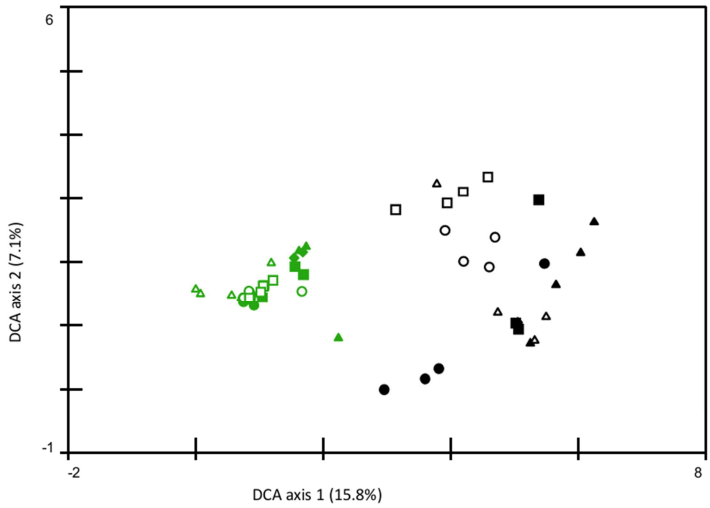

Figure 3 Detrended correspondence analysis of OTUs based on pyrosequencing of the *chiA* catalytic domain. Green symbols: grassland soil; black symbols: forest soil. Circle: crystal chitin; triangle: *Mucor hiemalis* fungal cell wall; diamond: *Aspergillus niger* cell wall; square: mealworm cuticle. Closed symbols: 3 weeks after inoculation; open symbols: 7 weeks after inoculation.

DCA analysis indicated that the composition of bacterial OTUs as based on *chiA* sequences was strongly affected by the soil origin (Figure 3). Forward selection in CCA showed that soil and time had the strongest effect on the development of *chiA* containing bacterial community ($p < 0.01$). Yet, also the effect of the chitin resource was significant ($p < 0.05$).

ChiA sequences assigned to beta- *Proteobacteria* were dominant at both harvests in all chitin-containing sand patches, except the *Aspergillus* cell wall treatment, in grassland soil (Table 6). When looking at narrower phylogenetic level most of the beta- proteobacterial *ChiA* sequences were assigned to the genus *Janthinobacterium* (Supplementary Table 3). Gamma-proteobacterial *ChiA* sequences which were also prominently present in most of the chitin treatments in the grassland soil patches were assigned to the genus *Stenotrophomonas* (supplementary Table 3). Actinobacterial *ChiA* sequences which were obtained

from all treatments were assigned to the genus *Streptomyces* (supplementary Table 3).

In the forest soil the dominating *chiA* sequences in several chitin-containing sand patches were assigned to gamma-*Proteobacteria*. Like for the grassland soil most of these sequences were assigned to the genus *Stenotrophomonas*, albeit that another genus*, Rhodanobacter*, was also strongly represented in some treatments (supplementary Table 3). *ChiA* sequences assigned to *Actinobacteria* were far more abundant in the sand patches in forest soils than in grassland soil and were even dominating in some treatments (crystalline chitin, harvest 1; mealworm cuticles, harvest 2). Almost all actinobacterial *ChiA* sequences were assigned to the genus *Streptomyces*. Another difference with the grassland soil was the strong increase of *chiA* sequences in the second harvest assigned to *Bacilli,* in particular to the genus *Paenibacillus* (Table 7, Supplementary Table 3). Most sequences of beta-proteobacterial *ChiA* genes, which were less abundant than in the grassland soil, were again assigned to the genus *Janthinobacterium*.

4. Discussion

4.1 Main factors determining bacterial community composition in chitin-enriched sand patches

Most studies dealing with chitin degradation in soil have used more or less purified chitin of marine arthropods (crabs, shrimps) as substrate (Sharma, 2013; Beier and Bertilsson, 2013). However, in soils the major natural sources of chitin are fungal cell walls and insect cuticles. The aim of the current research project was to study if terrestrial chitin-containing resources will be degraded by other microorganisms than the ones that are degrading the commonly used marine chitin resources.

We identified the bacteria that colonized patches of sand mixed with chitin-containing substrates from two different soils. For both the grassland and

forest soil the abundance of bacterial classes as based on *ChiA* gene sequences was in many cases significantly different between chitin-containing substrates. Hence, an effect of the different chitin resources could indeed be recognized. However, this effect was marginal when compared to the effect of the soil origin (Figure 3). In addition, also harvest time had a strong effect on the *ChiA* based composition of bacterial families, in particular for the forest soil. Effects of soils on composition of bacteria developing on added substrates have been shown often (De Ridder-Duine et al., 2005; Eichorst and Kuske, 2012). This has been attributed to the differences in the composition of indigenous bacterial communities between soils creating a difference in opportunities for bacterial species to colonize and/or degrade added substrates. In addition, differences in soil abiotic factors, like pH, may also influence the composition of substrate-colonizing bacteria (Kielak et al., 2013). In this study we used two sandy soils that were closely located to each other (1 km distance) but had a different history (former arable versus forest) and a clear difference in acidity and organic matter content. Hence, both indigenous community composition and soil characteristics may have had influence on the assembly of bacterial communities colonizing chitin-containing sand patches.

4.2 Dynamics of bacterial taxa in sand patches

Relative abundance of *chiA* sequences indicated that *beta-Proteobacteria* and *gamma-Proteobacteria* were rapidly responding groups of chitinolytic bacterial colonizers for both soils and for all chitin-containing substrates. Most of the C*hiA* sequences assigned to *beta-Proteobacteria,* which were the dominant patch colonizers in the grassland soil, showed the highest similarity with the genus *Janthinobacterium.* The genus *Janthinobacterium* (family *Oxalobacteraceae*) is well known for its chitinolytic activity and has often found to respond rapidly when purified or non-purified (e.g. shrimp waste) marine chitin resources were added to soils or soil slurries (Kielak et al., 2013; Wieczorek et al., 2014). Most of the beta-proteobacterial *ChiA s*equences retrieved from sand patches in the

forest soil were also assigned to *Janthinobacterium*. Several chitinolytic isolates belonging to the genus *Janthinobacterium* were obtained from sand patches buried in both soils, which is further supporting the stimulation of this genus by a range of chitin-containing substrates.

ChiA sequences assigned to the genus *Stenotrophomonas* were dominant among the gamma-proteobacterial *ChiA* sequences in chitin-containing patches in both the grassland and forest soils. An increase of *Stenotrophomonas* sequences has been reported for many studies were marine arthropod chitin resources have been incorporated in soils (Krsek and Wellington, 2001; Metcalfe et al., 2002; Kielak et al., 2013). Interestingly, *Stenotrophomonas chiA* sequences did not respond to the marine chitin resource (crystalline chitin) in patches buried in grassland soil, whereas a clear response was seen in the forest soil. The current study indicates that real terrestrial chitin resources, fungal cell walls and insect cuticles, do also have a strong stimulatory effect on the growth of *Stenotrophomonas* bacteria. For unknown reason, isolates identified as *Stenotrophomonas* were only obtained from the grassland soil.

Based on their reported abilities to degrade crystalline chitin and fungal remainders (Beyer and Diekmann, 1985; De Boer et al., 1999), their complex chitinolytic systems (Schrempf, 2001; Bai et al., 2015), and their abundance in chitin-amended soils (Metcalfe et al., 2002; Johnson-Rollings et al., 2014) we expected a dominance of actinomycetes in most treatments. However, relative abundance of actinomycetes, which were most represented by the genus *Streptomyces*, was low in most treatments for both soils. Two recent studies did also find a minor response of of *chiA* genes of actinomycetes as compared to those of proteobacteria after addition of partly purified shrimp chitin to agricultural soils (Kielak et al., 2013; Cretoiu et al., 2013). Kielak and colleagues (2013) suggested that chitinolytic proteobacteria were rapidly colonizing the chitin substrates and degrade the easily accessible parts of the chitinous substrate, whereas actinobacteria would become more important at later stages which were

not included in their study. Some of our results are in agreement with this suggestion, namely the general increase in relative abundance of putative streptomycetal *ChiA* sequences in the forest soils patches during the second harvest. However, the high relative abundance of actinomycetal *ChiA* genes at the first harvest in the sand patches containing crystalline chitin indicates that actinomycetes can respond quickly as well. In addition, it has been shown that crystalline chitin in sand microcosms can be degraded rapidly by actinomycetes (De Boer et al., 1999). Therefore, antagonism of rapidly colonizing chitinolytic proteobacteria against actinomycetes may be the most likely explanation for the general poor performance of the latter during initial degradation of chitinous substrates. The competitive advantage of rapid colonizing microbial species during initial stages of decomposition is a general phenomenon (Hibbing et al., 2010).

A similar scenario, namely prevention of invasion of sand patches by rapidly colonizing bacteria, may also explain the increase of *chiA* sequences assigned to bacilli, in particular to the genus *Paenibacillus*, at the second harvest in the forest soil. The increase of the genus *Paenibacillus* during prolonged incubation (2^{nd} harvest versus 1^{st} harvest) was also apparent from the isolation of several strains from 2^{nd} harvest patches. This is in contrast to actinomycetes for which no isolates were obtained. Most likely the short period of incubation (1 week) of the chitin-agar plates was not sufficient for development of actinomycetal colonies.

Comparison of chitinolytic isolates, *ChiA* sequences and 16S RNA gene sequences did point at potential shortcomings in the description of composition of chitinolytic bacterial communities as based on *ChiA* gene sequences. Most notably was the isolation of several chitinolytic bacteria belonging to genera *Flavobacterium* and *Chitinophaga* from sand patches incubated in grassland soil, without retrieving *ChiA* sequences from these genera in DNA extracted from the sand patches. Yet, the 16S ribosomal gene sequences indicated that phylum *Bacteroidetes* to which these genera belong was abundantly present in grassland

soil sand patches. This may point at a failure of the used *ChiA* primers to amplify *ChiA* genes from bacteroidetes. Indeed, annotation of the genome of *Chitinophaga pinensis* indicated that the chitinase-like genes of this bacterium showed little similarity with known chitinases (Glavina del Rio et al., 2010).

A similar discrepancy as for bacteroidetes was observed for pseudomonads. Several chitinolytic isolates were assigned to the genus *Pseudomonas*. Most of these were from sand patches incubated in grassland soil. However, all *chiA* sequences assigned to gamma-*Proteobacteria* had the highest similarity with *Stenotrophomonas* (family *Xanthomonadaceae*) and not with *Pseudomonas* (family *Pseudomonadaceae*). This is in contrast with the 16S RNA gene sequences which revealed a high abundance of both gamma-proteobacterial families in sand patches (Supplementary Table 1). It may be that the difference in the sequences of the catalytic domain of *ChiA* between Pseudomonas and *Stenotrophomonas* is not big enough of to assign these sequences to different species.

Based on 16S ribosomal sequence a strong increase of the classes alpha-*Proteobacteria* and *Acidobacteriia* was seen in the sand patches at the second harvest for the grassland- and forest soil, respectively. However, no or very low numbers of *ChiA* genes were retrieved that were assigned to these classes. In addition, with exception of 1 chitinolytic *Rhizobium* isolate, no chitinolytic isolates were obtained for these bacterial groups. This could imply that these groups were not involved in chitin-degradation but in degradation of other compounds or were cross-feeding (growing on oligomers released from polymers by extra-cellular enzymes of other microbes). With respect to the latter, it is interesting to note that chitinase genes are much less frequently seen for alpha-proteobacteria than for beta- and gamma-proteobacteria (Zimmerman et al., 2013). However, genes coding for degradation of chitin oligomers are widespread among alpha-proteobacteria making the possibility for cross-feeding realistic (Zimmerman et al., 2013). Chitinase genes have been detected in genomes of acidobacteria

(Zimmerman et al., 2013). However, since these bacteria are difficult to culture the diversity of chitinase genes in acidobacteria is not known. Hence, the apparent absence of *ChiA* sequences may not necessarily point at other activities than chitin degradation but may be due to lack of amplification with the used primer combination.

4.3 Evaluation of the experimental set-up.

As already indicated in the former section the actual dynamics of chitinolytic bacterial community composition in the chitin-enriched sand patches can be different from the results that were obtained on basis of *ChiA* sequences because of incomplete knowledge on the diversity of bacterial chitinases. In addition, it has been suggested that horizontal transfer of chitinase genes may interfere with taxonomic composition (Kielak et al., 2013).

The use of sterile chitin-enriched sand patches buried in soil rather than direct incorporation of chitin resources in soil may also have impacted the dynamics of chitinolytic bacteria. Bacteria have to invade the sand patches and, therefore, a selection towards the most motile chitinolytic bacteria could be expected. Differences in motility abilities can cause a selection of colonizers of sterile soil patches (Wolf et al., 2015). However, comparison of our results with those of Kielak and colleagues (2013), who analyzed *ChiA* sequences after addition of partly purified shrimp chitin directly to an agricultural soil, showed strong similarities in the responding and non-responding chitinolytic taxa. An advantage of using our set-up is that the bacteria recovered from the sand patches have to be active to colonize the chitin resources whereas direct extraction of DNA from chitin-amended soil will also reveal chitinase genes of bacteria that were present but not necessarily active. Indeed, no DNA could be extracted from sand patches without added chitin substrates indicating that the chitin-substrates were essential for proliferation of the invading bacteria.

An issue that we did not address in this study is the possibility that fungi can also have been involved in the degradation of the chitin resources in the buried sand patches. In an earlier study it has been shown that fungi can rapidly colonize and degrade chitin particles that were added to soil (De Boer et al., 1999). In that same study a competitive interaction for chitin between fungi and actinomycetes became apparent. Hence, interactions with chitinolytic fungi may have influenced the dynamics of chitinolytic bacterial community composition. It may be that the low response of chitinolytic bacteria to the presence of *Aspergillus* cell walls is due to a high activity of cell wall degrading fungi.

Despite these considerations the current study does point at important aspects on the dynamics of chitin-degrading soil bacteria upon addition of chitin resources:

(1) Soil origin has an overruling effect on the composition chitinolytic bacterial colonizers of chitin resources.

(2) There is no clear difference in the composition of responding chitinolytic bacteria when different marine and terrestrial chitin resources are added to soil.

(3) Chitinolytic *Proteobacteria* are rapidly responding to addition of chitin resources to soil.

Chapter 5

Impact of plant growth stage and fungal biomass on the composition of the chitinolytic bacterial community in the potato rhizosphere

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Abstract

Chitinases produced by bacteria in soil are involved in the degradation of chitin containing debris such as fungal remainders as well as in antagonistic activities against living fungi. Here, we tested the hypothesis that an increase in saprotrophic fungi in the rhizosphere will select for bacteria that use chitinases predominantly as inhibitors to compete with fungi for root exudates. In an earlier study fungal biomass was shown to increase in the rhizosphere of potato with maturation of plants in one agricultural site, but not in another site. In the current study, we followed the dynamics (abundance and diversity) of bacterial *chiA* genes in the rhizosphere samples of the same fields at different growth stages of potato. We observed that fungal biomass was not clearly coinciding with an increase of the abundance and diversity of bacterial *chiA* genes, but did coincide with changes in the composition of *chiA* gene harboring bacterial community. The relative abundance of the *chiA* harboring proteobacterial community increased significantly and the actinomycetal *chiA* harboring community decreased significantly with the increase of the rhizosphere fungal biomass during the growing season. The results support the hypothesis of fungal-induced selection of antagonistic chitinolytic bacteria as the dominant bacterial genera that responded to the increase in fungal biomass are *chiA* harboring *Proteobacteria*, in particular genus *Stenotrophomonas* and *Lysobacter*, which are well known for their antagonism against fungi.

Introduction

The immediate surroundings of the plant root, i.e. the rhizosphere, is a hot spot of (micro) biological activity in soil due to the continuous flow of energy-rich rhizodeposits from the root into the soil (Dennis et al 2010). It is generally thought that bacteria are the main users of the rhizodeposits, in particular of root exudates (Buee et al 2009, Dennis et al 2010). However, recent evidence showed that saprotrophic fungi can be as important as bacteria as primary consumers of root exudates (Buee et al 2009, Hannula et al 2012). Thus, when fungi are important as primary consumers of root exudates rhizosphere bacteria do not only need to compete with other bacteria, but they also need to compete with saprotrophic fungi (de Boer et al 2008). As a result, saprotrophic fungi in the rhizosphere could have selective influence on the bacterial community composition and functioning (de Boer et al 2008).

Bacteria are known to have a wide range of competitive strategies to antagonize fungi, including the production of inhibiting secondary metabolites and of cell wall lysing enzymes (Hibbing et al 2010). Among the latter, chitinases are of special interest as chitin is a structural component of the fungal cell wall (Bowman & Free 2006, Gooday 1994, Synowiecki & Al-Khateeb 2003). Thus, bacterial chitinases may be involved in weakening/degrading fungal cell walls, thereby inhibiting fungal activity and growth. This holds in particular for the hyphal tip, which is most vulnerable to chitinases as the chitin of the hyphal tip is in its native stage and not yet cross-linked with other cell wall polymers (Arlorio et al 1992, Bowman & Free 2006).

The occurrence of chitinase genes is taxonomically widespread among soil bacteria (Gooday 1990, Gooday 1994). However, the composition of the chitinolytic systems differs strongly between soil bacterial species with respect to the number of chitinase-encoding genes, their modular composition and the presence of chitin-binding proteins (Bai et al, 2015). For example, *Actinomycetes* which are well known for their ability to degrade organic polymers have, on

average, high numbers of chitinase genes containing different carbohydrate modules. The combination of this complex chitinolytic system, with the presence of other fungal cell wall lytic enzymes (*e.g.* β-1, 3-glucanases) and their hyphal growth form is probably the reason why *Actinomycetes* are known as important degraders of fungal remainders, which consist largely of cell walls. Several other soil bacteria have much simpler chitinolytic systems and often lack β-1, 3-glucanases (Bai et al 2015). It is questionable if such bacteria are able to degrade the chitin in fungal remainders. An example of such bacteria is the genus *Collimonas* that is known for its ability to obtain organic nutrients from living fungal hyphae. These so-called mycophagous bacteria can, however, only benefit from young fungal hyphae and stop growing on hyphae when the fungal mycelium becomes mature (De Boer et al 2001). It has been proposed that a combination of membrane destabilizing compounds and chitinases may cause leaking of nutrients from the hyphal tips (Leveau et al 2010). Similar combinations of inhibiting compounds and chitinases may also be used by rhizosphere bacteria to antagonize fungal competitors for root exudates (de Boer et al 2008).

The dynamics of the biomass of saprotrophic fungi in the rhizosphere can be followed by measuring ergosterol, a sterol present in fungal membranes (de Ridder-Duine et al 2006). An increase of ergosterol is largely pointing at an increase of saprotrophic fungi and not of arbuscular mycorrhizal fungi as these are devoid of this compound (Olsson et al 2003). Hence, by following the ergosterol concentrations in the rhizosphere in relation to the dynamics of the bacterial community an impression can be obtained of the impact of saprotrophic fungi on rhizosphere bacteria. In an earlier study by Hannula et al (2010), ergosterol concentrations were measured in the rhizosphere of potato (*Solanum tuberosum*) at two field sites during different plant growth stages. At one field, there was a significant increase of fungal biomass along with plant maturation, while in another field the fungal biomass did not change significantly. In the current study, we used DNA extracts from that previous study to screen for possible effects of the increase of saprotrophic fungal biomass on the abundance and diversity of

chitinolytic bacteria. We hypothesized that an increase of fungal biomass will coincide with an increase in the abundance of chitinolytic bacteria. In addition, we hypothesized that the responding chitinolytic bacteria will be the ones with relatively simpler chitinolytic systems as we assumed that the increased fungal biomass will in particular select for bacteria antagonizing living fungi. Screening of the abundance and diversity of *chiA* genes was chosen as proxy for the abundance and diversity of chitinolytic rhizosphere bacteria. *ChiA* genes code for chitinases belonging to the GH family 18 which is widespread among bacteria and have been reported to be the most abundant chitinase type in soil (Beier & Bertilsson 2013, Metcalfe et al 2002).

Material and methods

Site description and sampling

Potato rhizosphere soil samples were collected from agricultural sites Valthermond (VMD) and Buinen (BUI) in the Netherlands in 2008. Site VMD was characterized as a sandy peat soil (with 19 % organic matter content) and site BUI as a loamy sand soil (5% organic matter content). Both soils were slightly acidic (pH-water around 5). Further details on location, soil characteristics and fertilizer treatments are presented in an earlier paper (Hannula et al 2010). The potato (*Solanum tuberosum* L.) variety 'Karnico' was grown in the two fields in 4 replicate plots each containing 28 plants. Rhizosphere soil samples were collected at three different growth stages, namely seedling/young (EC30), flowering (EC60) and senescence (EC90). Rhizosphere soil per replicate plot was a pooled sample of 4 soil subsamples of randomly selected plants which was collected by brushing the roots. Ergosterol concentration of the samples is shown in Table 1. Total DNA from the rhizosphere samples was extracted using Power Soil DNA isolation kit (MOBIO Laboratories, Carlsbad, USA) according to manufacturer's instructions.

Table 1 Ergosterol concentration as indicator of fungal biomass in the potato rhizosphere of cultivar 'Karnico' at different plant growth stages in two fields (data from Hannula et al., 2010)

Ergosterol (mg/kg DW)	BUI field	Mark	VMD field	Mark
Young stage	3,15±1,44	A	4,32±0,49	a
Flowering stage	11,47±3,87	B	4,51±0,46	a
Senescent stage	17,62±9,20	C	6,42±2,85	a

DW: dry weight; different letters indicate significant differences ($P < 0.05$).

ChiA diversity analyzed by T-RFLP

Terminal Restriction Fragment Length Polymorphism (T-RFLP) was used to determine the richness and composition of bacterial *chiA* genes. Gene fragments of catalytic domains of GH family 18 chitinase were amplified from DNA samples using forward primer ChiA_F2 (5'-CGT GGA CAT CGA CTG GGA RTW YCC-3') 5' end labeled with 5'6-FAM and reverse primer ChiA_R2 (5'-CCC AGG CGC CGT AGA RRT CRT ARS WCA-3') (Hobel et al 2005). The PCR reactions were performed according to Hobel et al.(2004) with exception of the annealing temperature which was increased from 42 °C to 53 °C. The expected length of the PCR products is approximately 270 bp.

After verifying the size of the PCR products by agarose gel electrophoresis, the products were digested with restriction enzyme HaeIII (New England Biolabs, Ipswich, England) at 37 °C for 3 h together with an appropriate buffer and bovine serum albumin (BSA) supplied by the manufacturer, and then incubated at 80 °C for 20 min. to inactivate the enzyme (Hobel et al 2005). Negative (water) and positive controls (chitinase PCR product from *Streptomyces coelicolor* A3 (2) pure culture) were used in all steps of the T-RFLP procedure. Digested products were desalted and purified by ethanol precipitation after addition of 3 M sodium acetate (pH 5.2) with glycogen as a carrier molecule in a microtitre-plate format. TRFs were analyzed by an ABI 3130 capillary sequencer using GeneScane™ -500 LIZ (Applied Biosystems, Westburg, The Netherlands) as a size standard in order to identify the size of the fragments.

Quantitative PCR

ChiA gene copy numbers were quantified as previously described (Yergeau et al 2007), using primers GA1F (5'-CGT CGA CAT CGA CTG GGA RTD BCC-3')/GA1R (5'-ACG CCG GTC CAG CCN CKN CCR TA-3') to amplify the partial catalytic domain of GH 18 family chitinase (Williamson et al 2000). Quantitative PCR measurements were performed on the Rotor-gene 3000 PCR cycler (Westburg, Leusden, the Netherlands) using asymmetrical cyanine dye mix (SYBR Green, Qiagen, Venlo, the Netherlands). In order to determine the gene copy numbers of *chiA* (approximately 450 bp), a standard curve was created based on cloning of fragments of *chiA* of *Streptomyces coelicolor* A3 (2) using the pGEM-T Vector system (Promega, Wisconsin, USA). After purifying the plasmid a range of 10^1 to 10^8 copy numbers was made using Nanodrop 2000 UV-Vis Spectrophotometer (Thermo scientific, Massachusetts, USA).

Pyrosequencing of *chiA* catalytic domain

ChiA gene catalytic domains were amplified as previously described (Kielak et al 2013). Primers used were GA1F/GA1R (Williamson et al 2000). 50-µl reaction mixtures contained 5 µl of 10× PCR buffer with 25 mM $MgCl_2$, 0.2 mM deoxyribonucleotide triphosphates (dNTPs) mix, 2% dimethyl sulfoxide (DMSO), 20 µM of 10-bp barcoded GA1F and GA1R primers and 2.0 U of FastStart Taq DNA Polymerase (Roche Applied Science, Mannheim, Germany). Amplifications were performed using a Peltier 96-well thermal cycler (BIOzym,-Landgraaf, The Netherlands). The thermal cycling conditions started with a denaturation step (95°C for 5 min), followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and elongation at 72°C for 1 min, and finished by a final elongation step for 10 min at 72°C. PCR products were cleaned using a PEG precipitation method (Paithankar & Prasad 1991) and agarose gel electrophoresis was performed to check the right size of bands (approximately 450 bp). After this check, the concentration of DNA amplicons was measured by a Quant-iT PicoGreen dsDNA Assay kit (Invitrogen, Paisley, UK). The fluorescence reading was

measured on a microplate reader (excitation 485 nm and emission 528 nm), and the absolute amount of DNA was calculated according to a lambda DNA standard curve. Finally, the mixtures for each line were sent for pyrosequencing to Macrogen (Seoul, Republic of Korea).

Data analysis

Pyrosequencing data were analyzed using Mothur (Schloss et al 2009). Sequencing errors were reduced by flowgrams, and were trimmed to remove primers and barcodes. Shhh.flows command was applied in order to perform a denoising procedure. ChimeraUchime was used to remove potential chimeras. The remaining reads were translated into amino acid sequences. Sequences containing internal stop codons and unidentified amino acids due to sequencing errors were removed. Each translated sequence was used as a Blast-P query against a chitinase sequence database (2171 sequences) obtained from CAZy (Lombard et al 2014) using a 10^{-20} E value cutoff. The remaining amino acid sequences were aligned using MAFFT version 7 (Katoh & Standley 2013), together with the corresponding region of the reference sequences. Distance matrix of aligned sequences was calculated by the protdist program of PHYLIP 3.67 package (Felsenstein 2005). The obtained matrix was applied to Mothur for clustering using average neighbor algorithm. Sequences were then assigned to operational taxonomic units (OTUs) based on a 5 % dissimilarity cutoff. This 5 % cutoff was chosen based on slope stabilization by plotting the number of unique OTUs at different OTU cut-off values. Bacteria that could be associated with *chiA* genes were identified based on BLAST-P analysis (threshold: e value of 10^{-20}) available at the National Center for Biotechnology Information website (NCBI).

T-RFLP data were visually inspected in Gene-Mapper Software v4.1 (Applied Biosystems, Westburg, the Netherlands) and then transferred to T-Rex for binning and filtering of the peaks in order to remove noise (Culman et al 2009). The lower threshold for fragment length was 60 bp; and upper threshold 245 bp; and a fluorescent threshold of 50 was used (Hjort et al 2010). Only presence-

absence data were used in the subsequent analysis. Nonmetric Multidimensional Scaling (NMDS) with Jaccard as distance measurement in PAST was used to assess the similarity of the chitinase-derived TRFs in treatments (Hammer et al 2001). The effects of field site and potato growth stage on abundance of *chiA* gene were assessed by two-way ANOVA with SPSS for Windows (19.0).

ANOSIM (Analysis of Similarity) analysis of *chiA* gene TRFs patterns was carried out in PAST as well. Numerical data, such as relative abundance of bacterial species within the community were analyzed with SPSS for Windows (19.0). Normality of data was checked with the Shapiro–Wilk's test. An appropriate transformation was applied for the data which were not normally distributed. Homogeneity of variances was assessed by Levene's test. Two-way ANOVA analysis of data was done by univariate analysis of variance in SPSS windows (19.0) to identify the differences among samples. Differences within numerical data with unequal variance were assessed with Kruskal-Wallis nonparametric tests. Correlation between different parameters was calculated using linear regression models in PAST (Hammer et al 2001)**.**

Results

Diversity of bacterial *ChiA* genes determined by T-RFLP

ANOSIM analysis (Table 2) revealed that the *chiA* TRFs profiles significantly differed between the two fields (Figure 1, $p < 0.005$). In VMD field, the TRFs of young stage significantly differed from flowering and senescent stages (Figure 1, $p < 0.05$). In the BUI field, the TRF patterns were more scattered, indicating more variation between plots and no significant differences between growth stages were detected.

Table 2 Similarity analysis (ANOSIM) on *chiA* TRFs pattern in the rhizosphere of potato plants in 2 arable fields measured at different plant growth stages. Values represent the statistic R for one-way ANOSIM in PAST. Lower R means less difference between different groups. All ANOSIM analyses were performed using Bray-Curtis index and 10 000 permutations. Significant mark of R: **p<0.005 and *p<0.05 (P-value: sequential Bonferroni significance P value)

	One-way ANOSIM
	ChiA TRFs profile pattern (HaeIII)
Soil (BUI vs. VMD)	0,196**
Growth stages	
Field BUI	
Young stage vs. Flower stage	0.3594
Young stage vs. Senescent stage	0.3802
Flower stage vs. Senescent stage	-0,1771
Field VMD	
Young stage vs. Flower stage	0.7813*
Young stage vs. Senescent stage	0.7396*
Flower stage vs. Senescent stage	0.3073

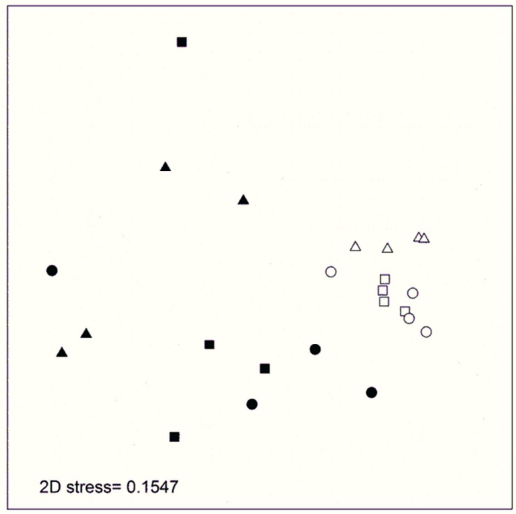

Figure 1 NMDS ordination plot of *chiA* TRFs profiles in potato rhizospheres as obtained after restriction with HaeIII. BUI field: filled symbols; VMD field: open symbols. Triangles: young stage, squares: flowering stage, circles: senescent stage of the potato growth

Abundance of bacterial *chiA* genes

Quantitative PCR results indicated that plant growth stage was the main factor influencing the *chiA* gene copy numbers (Figure 2). In field VMD *chiA* copy numbers increased significantly ($p < 0.05$) from young to senescent stages, while in field BUI the highest copy numbers were measured at the stage of flowering.

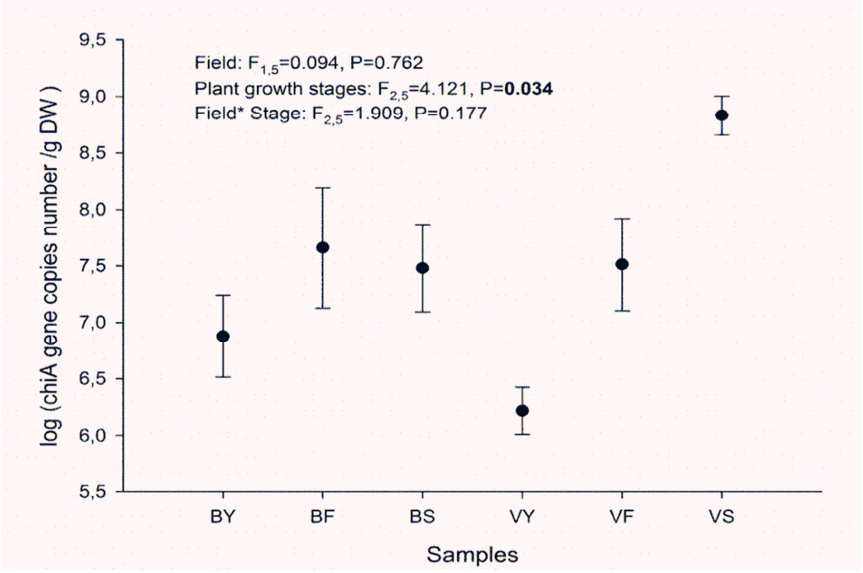

Figure 2 Quantitative PCR based abundance of bacterial *chiA* gene copy numbers in the rhizosphere of potato plants in 2 arable fields measured at different plant growth stages. Error bars represent standard errors of the mean of four replicate plots. B: BUI field; V: VMD field. Y: young stage; F: F: flowering stage; S: senescent stage

Correlation with fungal biomass (ergosterol)

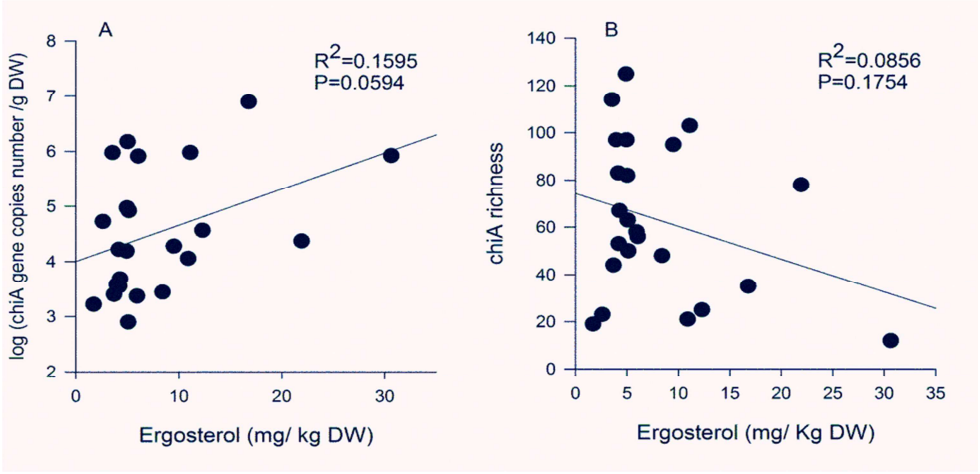

Figure 3 Correlations between rhizosphere fungal biomass (ergosterol) and numbers of bacterial *chiA* gene copies (A) and with diversity (richness) of bacterial *chiA* genes according to T-RFLP method

In the study of Hannula et al (2010) ergosterol was used as a proxy for the fungal biomass. As shown in figure 3, the ergosterol content in potato rhizosphere was not significantly correlated with *chiA* gene copy numbers, albeit that the P-value was close to the 5% significance level. The richness of *chiA* expressed in numbers of *chiA* TRFs was not correlated with the amount of ergosterol.

Pyrosequencing of the catalytic domain of bacterial *chiA*

On average 542 sequences of *chiA* gene fragments per replicate plot passed the quality filtering. These sequences revealed information on the taxonomic composition of the bacteria harboring *chiA* genes. Richness and diversity of these sequences for each sample are shown in table 3. The identified best hit and relative abundance of bacteria from each sample is shown in figure 4.

Table 3 Richness and diversity estimator of pyrosequencing of *chiA* catalytic domain

	Average score ± Standard Error			
	Richness estimator		Diversity index	
Sample	Sobs[a]	Mark	Shannon	Mark
BY	85±2	A	3,95±0,02	A
BF	83±5	A	3,89±0,09	A
BS	89±3	A	3,91±0,05	A
VY	87±2	a	4,02±0,06	a
VF	105±3	b	4,36±0,03	b
VS	90±3	a	4,10±0,05	a

[a]Observed richness. Different letters indicate significant differences (P < 0.05). (B: BUI field; V: VMD field. Y: young stage of potato growth; F: flowering stage of potato growth; S: senescent stage)

Table 3 shows that in the BUI field, the richness and diversity of *chiA* did not differ significantly between plant growing stages. However, in VMD field, *chiA* richness and diversity at the flowering stage significantly differed from the young and senescent stages ($p<0.05$). As for the T-RFLP analyses, there was no significant correlation between ergosterol (= fungal biomass) and the richness and diversity of the *chiA* harboring bacterial community, in neither field.

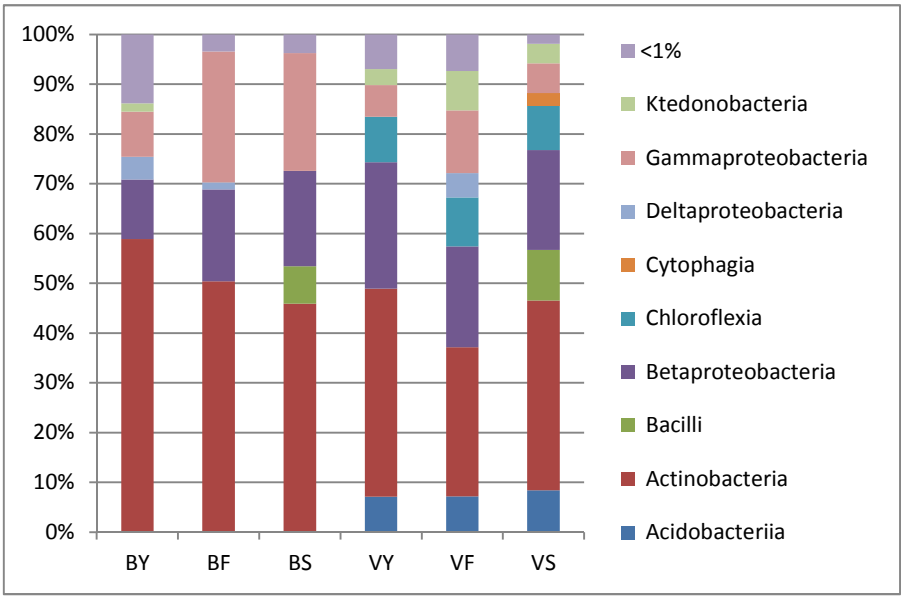

Figure 4 Relative abundance of bacterial classes as based on pyrosequencing of the *chiA* catalytic domain (BLAST-P analysis). Calculations were performed on normalized data. Relative abundances are the average of four replicate plots per field site and plant growth stage. Bacteria that have an abundance of less than 1% of the sequences are pooled and presented as "<1%" group. (B: BUI field; V: VMD field. Y: young stage of potato growth; F: flowering stage of potato growth; S: senescent stage)

Identification of *chiA* gene fragments indicated that the composition of the bacterial community harboring *chiA* genes differed between fields as well as between plant growth stages. *ChiA* genes assigned to the classes *Actinobacteri* and, beta- and gamma- *Proteobacteria* are dominant in all samples. However, their relative abundance varied with plant growth stages. For instance the relative abundance of *chiA* genes of the class *Actinobacteria* was significantly higher in the young growth stage compared to the other stages in both fields (Figure 5). The relative abundance of *chiA* genes of beta- and gamma-*Proteobacteria* was lowest in the young plant growth stages at BUI field. However, at the VMD field this was not the case for beta-*Proteobacteria*. Remarkably *ChiA* genes assigned to classes *Acidobacteria* and *Chloroflexia* were present in the VMD field at all plant growth

stages, whereas they were not detected at any growth stage in the BUI field. *ChiA* genes assigned to the class of *Bacilli* were only detected in senescent growth stages at both fields (Figure 4).

The *chi*A-gene abundance of some well-known chitinolytic genera was analyzed separately. *Streptomyces-chiA* genes were abundant in the potato rhizosphere in both fields. Interestingly, for both fields a significant decrease in *Streptomyces- chiA* genes was observed with increasing maturity stages of the potato plants (Figure 6). In contrast, for two well-presented genera within the gamma-*Proteobacteria*, i.e. *Lysobacter* and *Stenotrophomonas-*, the relative abundance of the *chiA* gene was lowest in the young potato stage and increased in later growth stages. Frequencies of chitinases assigned to the genus of *Janthinobacterium*- (beta-*Proteobacteria*) showed opposite patterns for the two fields (Figure 6). It increased with maturity of the plants at the BUI field and decreased at the VMD field.

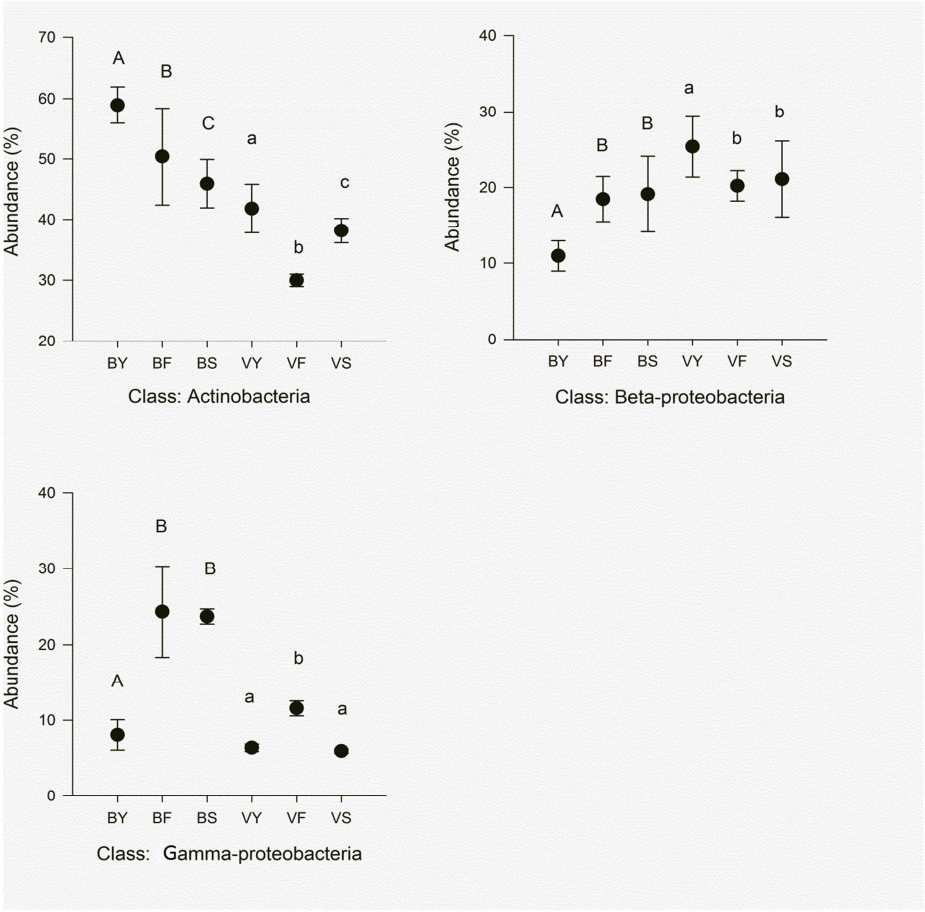

Figure 5 Relative abundance of *chiA* sequences of dominant bacterial classes in the potato rhizosphere at different plant growth stages. B: BUI field; V: VMD field. Y: young stage; F: flowering stage; S: senescent stage. Different letters above growth stages indicate significant differences ($P < 0.05$)

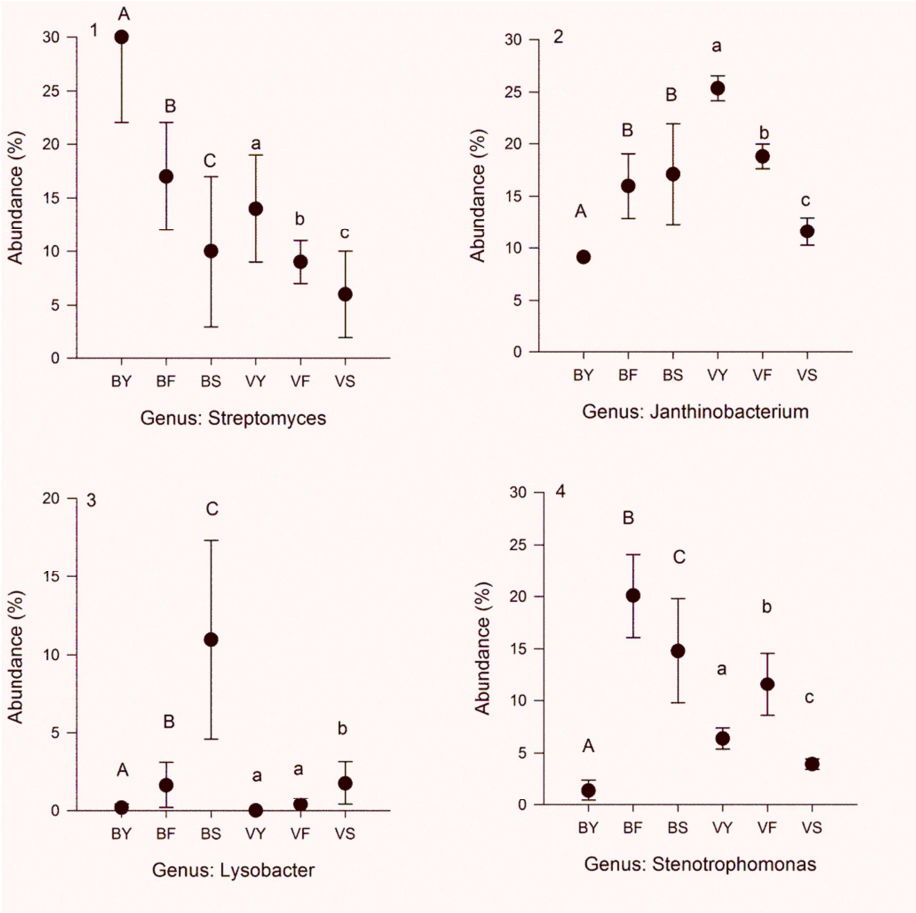

Figure 6 Relative abundance of *chiA* sequences of dominant bacterial genera in the potato rhizosphere at different plant growth stages. B: BUI field; V: VMD field. Y: young stage; F: flowering stage; S: senescent stage. Different letters above growth stages indicate significant differences ($P < 0.05$)

Discussion

It has been suggested that bacterial chitinases represent an important part of the "antagonistic toolbox" with which bacteria can attack competing fungi (de Boer et al 2008). This function of bacterial chitinases is also evident from the fact that many bacteria known as potential biocontrol agents of root-infecting

fungi show chitinolytic activity (Kobayashi & Crouch 2009). In addition, direct inhibiting effects of purified bacterial chitinases on the development of hyphae of pathogenic fungal hyphae has been shown (Hjort et al 2014). On the other hand, bacterial chitinases may play a role in the degradation of fungal remainders in soil. These remainders consist mainly of cell walls containing chitin cross-polymerized with other polymers (Bowman & Free 2006).

Our study revealed changes in numbers and diversity of chitinolytic bacteria in the rhizosphere of potato during the growth season. Older plant growth stages had more chitinolytic bacteria in the rhizosphere than young plants and this coincided with a higher relative abundance of *Proteobacteria* and a lower relative abundance of *Actinobacteria*.

Our hypothesis was that an increase in numbers as well as a change in the composition of the chitinolytic bacterial community would be caused by an increase in abundance of saprotrophic fungi. Ergosterol, as a proxy of the saprotrophic fungal biomass, concentrations increased during the growth season, albeit that the increase was most prominent and significant at site BUI. We expected that an increased saprotrophic fungal biomass would especially select for bacteria that use chitinases for antagonistic interactions with living fungi. A positive correlation between the qPCR-based abundance of bacterial *chiA* genes and ergosterol concentrations was nearly significant, supporting the fact that the saprotrophic fungal biomass in the rhizosphere might be an important factor steering the numbers of chitinolytic rhizosphere bacteria. Pyrosequencing analysis of the catalytic domains of *chiA* gene showed that fungal dynamics had no effect on *chiA* gene richness and diversity. However, it had an effect on the *chiA* harboring bacterial community composition. The analysis showed an increase in relative abundance of *chiA* genes of gamma- and beta-*Proteobacteria* and a decrease of *chiA* genes of *Actinomycetes* which coincided with a strong increase of the fungal biomass in the rhizosphere in the BUI field. *Actinomycetes* have been indicated to be major chitin degraders in soil (Krsek & Wellington 2001). The

complex chitinolytic systems and possession of other lytic enzymes that is known from many *Actinomycete* genomes may be essential to access and degrade chitin in fungal cell walls; in contrast, the chitinolytic system of many *Proteobacteria* is less complex (Bai et al 2015). Therefore, it has been suggested that these bacteria may use chitinases preferentially to suppress living fungi during competition or to feed on living fungi (De Boer et al 2001). Hannula et al (2012) followed a pulse of labeled $^{13}CO_2$ through mature potato plants (*Solanum tuberosum*) and showed that rhizosphere fungi belonging to the phylum *Ascomycota* were rapidly incorporating recently fixed plant carbon. This relative importance of fungi as primary consumers of root exudates will impose a substantial competitive stress on rhizosphere bacteria. Therefore, the strong increase during the growth season of chitinolytic *beta-* and *gamma-Proteobacteria*, which are known to be typical rhizosphere dwelling organisms, with that of fungal biomass (ergosterol) in the BUI field may point to a prominent role of chitinases in antagonistic activities of bacteria against fungi. This is even more supported by the fact that *Chi*A genes of the proteobacterial genera *Stenotrophomonas* and *Lysobacter* were among the most responsive ones. These genera are known for their antagonistic interactions with fungi, and several strains are being or have been tested for their potential to suppress root-infecting fungi (Jankiewicz et al 2012, Ryazanova et al 2005). The decrease in the relative abundance of *Chi*A genes of *Actinomycetes* may suggest that these filamentous bacteria do not compete with fungi for root exudates. Inceoglu et al (2011) analyzed bacterial 16S rDNA fragments in the same samples and observed that *Actinobacteria* with the dominant genus *Streptomyces* showed a decreasing pattern in the potato rhizosphere from young to senescent stages. Yet, *Actinomycetes* are known for their antifungal activities as well (Quecine et al 2008, Saxena et al 2013). However, given the known capacity of *Actinomycetes* to degrade organic polymers, it seems more likely that they compete with fungi for such compounds rather than for root exudates.

Chapter VI

General discussion

The objective of this study is to get a better understanding of bacterial chitinases and, in particular, to address the factors, in particular the presence of fungi, that influence the functioning of the enzymes. Ecologically, bacterial chitinases are of prime importance in terrestrial ecosystems as they are main enzyme systems involved in the degradation of chitin, which is one of the most abundant polymers in soils. In addition, chitinases form a key component in the interaction of bacteria with fungi, as they may be used to attack fungi by destabilizing the fungal cell walls in which chitin is an important structural component. The latter is particularly important in terrestrial environments where both bacteria and fungi are the dominant micro-organisms.

This study addressed the following research questions: 1) Can the functioning of bacterial chitinases be explained by the composition of the chitinolytic system and/or phenotypic characteristics?; 2) Is there evidence for the importance of bacterial chitinases in competitive interactions with fungi?; 3) Is there differentiation among chitinolytic bacterial species with respect to degradation of different chitinous organic sources?

In this chapter I will discuss the results of the study from an ecological perspective and I will give suggestions for further future research on and applications of bacterial chitinases.

Can the functioning of bacterial chitinases be explained by the composition of the chitinolytic system and/or phenotypic characteristics?

Chitinolytic systems include chitinases, chitin binding proteins and other proteins that make chitin accessible to chitinases. In addition, morphological or

motility characteristics of bacteria can have an important contribution to their chitin-degrading performance, for instance with respect to the possibilities of bacteria to get access to the chitin polymers. At the start of research described here it was already known that there is a huge variation among chitinolytic bacteria with respect to their chitin degrading performance and antifungal activity (de Boer et al 2008, De Boer et al 1999). This led to the hypothesis that the functioning of bacterial chitinases is related to the composition of the chitinolytic system.

In chapter 2, I used an in *silico* approach, in order to compare the genomics of the chitinolytic systems of all publicly available bacterial genomes with data on the ecology of those bacteria. In particular, chitinolytic systems of aquatic and terrestrial bacteria were compared. One of the major reasons for differences in chitinolytic systems of aquatic versus terrestrial bacteria is the nature of the available chitin resources. In aquatic systems, chitin is mainly present in the exoskeleton of arthropods while in terrestrial habitats chitin is mainly found as components of fungal cell walls. Moreover and in line with the nature of the available chitin resources chitinases of terrestrial bacteria may also play a role as antifungal tool in the interaction between bacteria and fungi (Aktuganov et al 2008, Arlorio et al 1992). Thus, in soil the functioning of bacterial chitinases could be more complex than in aquatic ecosystems and this may lead to a more complex or, at least different, composition of the chitinase enzyme system of soil-borne versus aquatic bacteria. The results of chapter 2 showed, indeed, that the chitinolytic system of terrestrial bacteria has a rather complex structure and the potential to perform multiple functions. The screening of the modular composition of chitinases in bacterial genomes indicates differences between aquatic and terrestrial bacteria that could be related to the different chitinous resources they utilize. For instance, terrestrial *Actinobacteria*, which are known to be highly capable of decomposing chitin polymers, do contain more diverse chitin binding domains than the aquatic *Actinobacteria*. Terrestrial *Proteobacteria* and *Bacilli* possess more polyketide synthase genes which code for enzymes involved

in the production of antibiotic compounds in association with chitinase complexes, which indicates the specific use of the chitinases for antifungal activities by these bacteria. Overall, I observed less complex chitinolytic systems for aquatic bacteria than for terrestrial bacteria. So, these findings support the aforementioned hypothesis.

Chitin binding proteins are functional components of the chitinolytic system common in bacteria (Vaaje-Kolstad et al 2005a, Vaaje-Kolstad et al 2010). I found that the degradation rate of crystal chitin was significantly correlated with the number of chitin binding proteins (chapter 3). Thus, chitin binding proteins may be an important factor of the chitin degrading capability of bacteria. This may also explain the pronounced ability of *Actinomycetes* to degrade crystal chitin as shown in chapter 3, as these organisms have a larger variety of chitin binding domains than most other bacteria as I mentioned earlier.

Another factor mentioned to be of importance for the chitin degrading capabilities of bacteria is their morphological structure. Hyphal forming bacteria, such as *Actinomycetes*, are reported to be better capable of degrading chitin particles than most non-hyphal bacteria (De Boer et al 1999). Filamentous bacteria have often been found to be the dominant chitin-degrading bacteria when chitinous material was added to soil (Krsek & Wellington 2001, Sato et al 2010). One of the possible reasons for the good chitin degrading abilities of *Actinomycetes* might be that the hyphae can penetrate the chitin particles thereby extending the area where chitinases are in close contact with the chitin polymers. However, the results of chapter 3 did not support this as I did not found a correlation between bacterial morphology and rate of degradation of different chitin resources, neither were filamentous properties of the tested bacteria related to antifungal activity.

Is there evidence for the importance of bacterial chitinases in competitive interactions with fungi?

Interactions between bacteria and fungi are recognized to be of great significance for the functioning of terrestrial ecosystems (Ingham et al 1985). Chitinases are believed to be important components in this interaction since they are keys to the decomposition of fungal remnants by bacteria as well as to the competitive abilities of bacteria as the chitinases can also decay the chitin of the fungal cell walls (De Boer et al 2005). Chitinases may also play a role in obtaining nutrients from living fungi. The latter is called bacterial mycophagy and has been examined in detail for soil bacteria of the genus *Collimonas* which can only degrade easily accessible chitin (Mela et al 2011). Therefore I hypothesized that chitinases and the chitinolytic capabilities of bacteria are related to fungal abundance or biomass

In order to test this hypothesis, I determined the *chiA* harboring bacterial community in the rhizosphere of potato plants during a full growing season along with the dynamics of fungal biomass by a metagenomic approach. The rhizosphere is a hot spot of biological activity and interactions. Fungi and bacteria are among the most abundant inhabitants of the rhizosphere and they interact strongly (Bell et al 2014, Berg et al 2006). Therefore I expected that the dynamics of the fungal biomass in the rhizosphere had a strong impact on the abundance of bacterial *chiA* genes (chapter 5).

The results described in chapter 5 gave some support to the hypothesis, that bacterial chitinases play a role in bacterial-fungal interactions. *ChiA* gene copies number increased proportionally with the saprotrophic fungal biomass in the potato rhizosphere and the composition of the *ChiA* gene harboring bacterial community was affected by the fungal biomass dynamics. Furthermore, the increase in fungal biomass coincided with the increasing abundance of *chiA* harboring β- and γ- *Proteobacteria* which fit to the concepts on the ecological role of their chitinolytic system as discussed in chapter 2, namely that these bacteria use, to a large extent, their chitinase system for anti-fungal activities. However, in contrast to the information available from several previous studies, e.g. (Glick

2012), Quecine et al (2008), Singh et al (2014) and the results of chapter 3, I observed a decreasing abundance of *Actinobacteria,* including *Actinomycetes* with increasing fungal biomass. The explanation could be that, in this case, *Actinobacteria* do not compete with fungi for root exudates, but that they use their chitinases mainly to compete for and to degrade chitinous material and/or other polymers in soil.

Is there differentiation among chitinolytic bacterial species with respect to degradation of different chitinous organic sources?

As one of the most abundant natural polymers, the degradation of chitin is a major ecological issue and subject of many studies reported in literature. The role of bacterial chitinases in the degradation of chitin has, in most of cases, been studied using shrimp or crab remainders as chitin sources (De Boer et al 1999, Jacquiod et al 2013, Johnson-Rollings et al 2014, Kielak et al 2013, Krsek & Wellington 2001). However, it is clear that this is a rather artificial approach and therefore, in my study on the responses of the bacterial community to the availability of different chitin resources, I compared the responses of the bacterial communities of two different soils, i.e. a grassland and a forest soil, to 4 different chitin resources as substrates, including chitin made from shrimp shell, two types of fungal cell walls and cuticles from mealworms. To our knowledge this was the first time that dead fungal hyphae were used as chitin resource in a chitin degradation study.

The results revealed an overruling effect of the original soil (= native bacterial community) on the bacterial community colonizing the different chitin resources. However, in each soil the effect of the chitin sources was also significant. As a response to the availability of the fungal chitin a different bacterial chitinolytic community was selected as compared to the availability of other chitin resources. Especially the absence of a chitinolytic bacterial

community in the treatment with chitin from *Aspergillus niger* indicates that bacteria may not be the main degraders of chitin from the cell wall material of this fungus.

The dominant bacterial classes selected in all treatments and in both soils were gamma- and beta- *Proteobacteria* which does not fit to the conceptual ecological role of the chitinolytic properties of these bacteria namely as competitive, antifungal tools (chapters 2 and 3). However, a legitimate explanation may be that the presumed r-strategy behavior and large motility of *Proteobacteria* enabled them to quickly colonize the chitin resources, whereas in the later stages, slower responding, K-strategists, such as *Actinobacteria* and *Bacilli* may become more abundant. Motility of bacteria could well be a significant advantage to bacteria colonizing chitin resources in natural soil conditions, which does not play a role in laboratory studies with artificial media such as the one described in chapter 3. Interestingly, I showed that *Bacilli* played an important role in the chitin degradation in forest soils, next to *Actinomycetes* and *Proteobacteria.*

I used two approaches to determine the selection of the bacterial community due to the availability of different chitin resources. The aforementioned results and conclusions were made on the basis of observations on the total community found in the bags containing the chitin resource. This means that other additional components also present in the natural chitin resources could have been a factor determining the community selection process. The other approach, determining the *chiA* harboring community is more specific and was also applied by Cretoiu *et al* (2013). They found different results at the level of genera of *chiA* harboring bacteria selected by the chitin resources, but, overall, the conclusion is justified that the study of *chiA* harboring bacterial communities is a legitimate approach to assess the chitinolytic bacterial community in ecosystems.

Perspectives for future research and applications

Based on the experiences of the current study, I suggest the following topics to be of importance for improvement of our knowledge of the microbial chitinolytic system and its role in ecosystem functioning:

- The composition of the chitinase enzyme complex, and in particular, the chitin binding proteins needs more attention.
- Until now, the focus has mainly been on *chiA* as indicator for chitinase due to the availability of specific primers and existing database. However, there are other types of chitinases reported to function in the bacterial chitinolytic system (Vaaje-Kolstad et al 2013). One of these is *chiB* (Igarashi et al 2014, Orikoshi et al 2005) which may be an important subject for further understanding of bacterial chitinases.
- This study showed that *Actinomycetes* prefer to use chitinases for chitin degradation. However, from other studies it is known that they have antifungal capabilities as well. Other bacteria, such as beta- and gamma-*Proteobacteria* were shown to prefer to use their chitinolytic system for antifungal activity, but they are also known as degraders of chitin resources. So, it is important to get a better understanding of the conditions under which these bacteria use their chitinolytic capabilities for different purposes.

Some useful applications can also be derived from the results of the current study:

- Chitin amendment (chitin from shrimp and crab cuticles) has been used as biocontrol strategy (Cretoiu et al 2013). The idea behind this approach is that by adding chitin resources to soil chitinolytic bacteria will be selected that may control pathogens such as plant pathogenic fungi. The current study points to the fact that the selection of the chitinolytic bacteria colonizing these materials is highly dependent on the indigenous community of the soil and that the selected bacteria are not necessarily antagonistic against fungi. Hence, inoculation of chitin resources together with chitinolytic antagonists

such as *Actinomycetes* or specific *Proteobacteria* may be an interesting and more effective application.

- The current study also showed several chitin binding domains within the bacterial chitinase complex that were seldomly described (chapter 2). Further studies on these domains may be of interest to detect novel chitinase functions.

Summary

Chitin is the second most abundant polymer in nature. In aquatic ecosystems, chitin is mainly present as part of the exoskeleton of arthropods; in soil, chitin is mainly present as component of fungal cell walls. The main enzymes involved in the breakdown of chitin are chitinases. Chitinases are produced by both fungi and bacteria. Chitinases of soil-borne bacteria can decompose chitin of dead fungal hyphae and other resources, but they may also play a role in antagonistic activities against fungi by destroying the chitin in the fungal cell walls. In the research described in this thesis, I tested the hypothesis that bacterial chitinases may perform different functions in different environments and under different circumstances, while the genetic composition and function of bacterial chitinases vary between different habitats.

Different approaches were applied in the current Ph D project including “in silico” genomic comparison of bacterial chitinolytic system as well as experiments. The results of genomic comparison of the chitinolytic system of terrestrial and aquatic bacteria in chapter 2 showed that terrestrial bacteria have more complex chitinolytic systems than aquatic bacteria which may be the result of adaptation to more complex functioning of chitinases in terrestrial habitats. In terrestrial ecosystems, bacterial chitinases may be involved in the degradation of chitinous material and in antifungal activity whereas in aquatic ecosystems chitinases mainly function as chitin degrading agents because in these systems the fungal biomass is generally low. In correspondence with these findings we observed more diverse chitin binding domains within the chitinase complex of terrestrial bacteria. Besides, I found a higher fraction of chitin binding proteins and other proteins that may be involved in antifungal activity within the genomes of terrestrial chitinolytic bacteria.

In chapter 3, a fungi-bacteria confrontation experiment using 13 different bacteria was conducted in order to investigate the effect of chitinase numbers

and morphological properties of bacteria on their chitin degradation capability and antifungal activity. Remarkably, we found that the number of chitin binding proteins within bacteria was significantly correlated with the capability to degrade crystal chitin. On the contrary, chitinase numbers and morphological properties of bacteria (hyphal structure versus single cells) were not correlated with any chitin source degradation or antifungal activity. This confirmed the importance of chitin binding proteins in crystal chitin degradation.

In order to find out if different bacterial communities are selected by different chitin sources, I added four different chitin resources including crystal chitin, two different types of fungal cell walls and cuticles from mealworm as substrates to two soils. Pyrosequencing of 16S rRNA of bacteria and of the catalytic domain of the bacterial *chiA* gene was applied to identify chitin-degrading bacteria containing chitinases. Both the composition of the total bacterial community and of the chitinolytic bacterial community was significantly affected by soil, chitin sources and time of incubation. However, the richness and diversity of the chitinolytic bacterial community were not different between chitin resources indicating that the chitin content of the material does not have a major effect on the relevant bacterial community as long as the chitin has an equivalent structure.

I also tested the *chiA* gene diversity and abundance, as well as the dynamics of the chitinolytic bacterial community in response to the dynamics of the saprotrophic fungal biomass in the potato rhizosphere in order to test the function of bacterial chitinases as tool in the competition with fungi. The results showed that an increase of the fungal biomass in the potato rhizosphere caused an increase of *chiA* copy numbers, an increasing relative abundance of beta- and gamma- *Proteobacteria*, and a decreasing abundance of *Actinobacteria*, indicating that chitinases do play a role in bacteria-fungi interactions in soil.

The results obtained in this study have contributed to a better understanding of the ecological functions of bacterial chitinases. New insights in the composition of the bacterial chitinolytic system and the importance of its components were

obtained. The potential ecological functions of the bacterial chitinase complex were explored and the role of chitinases in bacteria-fungi interactions, which are vital to the functioning of terrestrial ecosystems, was revealed further.

Samenvatting

Chitine is het op één na meest voorkomende polymeer in de natuur. In aquatische ecosystemen komt chitine voornamelijk voor als deel van het exoskelet van arthropoda; in de bodem komt chitine vooral voor als onderdeel van de celwand van schimmels. De belangrijkste enzymen, die betrokken zijn bij de afbraak van chitine, zijn chitinases. Chitinases worden zowel door schimmels als bacteriën geproduceerd. Chitinases van bodem gebonden bacteriën kunnen niet alleen chitine van dode schimmel hyphen en andere bronnen afbreken, maar zij spelen waarschijnlijk ook een rol in antagonistische activiteiten door het afbreken van de chitine in de celwanden van (levende) schimmels. In het onderzoek dat in dit proefschrift beschreven is, heb ik de hypothese getoetst dat bacteriële chitinases verschillende functies hebben in verschillende milieus en onder verschillende omstandigheden en dat de sequentie van het deel van het bacteriële genoom dat codeert voor chitinases en de functie ervan varieert tussen verschillende habitats.

In het onderzoek zijn verschillende benaderingen toegepast, waaronder "*in silico*" genoom vergelijking van bacteriële chitinase systemen en experimentele benaderingen. De resultaten van het vergelijkend genoom onderzoek van chitinolytische systemen van aquatische en terrestrische bacteriën in hoofdstuk 2 laten zien dat terrestrische bacteriën complexere chitinolytische systemen hebben dan aquatische bacteriën wat waarschijnlijk het gevolg is van adaptatie aan de complexere functies van chitinases in terrestrische habitats. Zoals eerder aangegeven zijn bacteriële chitinases in terrestrische systemen betrokken bij zowel de afbraak van chinine bevattend materiaal als bij antischimmel activiteiten terwijl in aquatische ecosystemen chitinases voornamelijk functioneren als chitine afbrekende agentia omdat in deze laatste ecosystemen de schimmel biomassa over het algemeen gering is. Bovendien, vond ik in de genomen van terrestrische bacteriën een grotere fractie aan coderingen voor chitine bindende proteïnen en

voor andere proteïnen die mogelijkerwijze betrokken zijn bij antischimmel activiteiten.

In hoofdstuk 3 wordt een schimmel-bacterie confrontatie experiment beschreven, met 13 verschillende bacteriën waarin het effect van het aantal chitinases per bacterie en morfologische eigenschappen van de bacteriën op hun vermogen tot het afbreken van chitine en antischimmel activiteit werd onderzocht. Het was opmerkelijk dat het aantal chitine bindende proteïnen statistisch significant correleerde met het vermogen tot het afbreken van kristallijn chitine. Daarentegen was er geen significante correlatie tussen het aantal chitinases per cel en morfologische eigenschappen, dwz hyphen structuur versus enkelvoudige cellen, van de onderzochte bacteriën met het vermogen tot afbraak van de verschillende chitine bevattende materialen of antischimmel activiteit. Dit bevestigde het belang van de chitine bindende proteïnen bij de afbraak van kristallijn chitine.

Om na te gaan of verschillende bacterie gemeenschappen worden geselecteerd door verschillende chitine materialen aan te bieden , heb ik vier verschillende chitine bevattende materialen , dwz kristallijn chitine , twee verschillende typen schimmel cel wanden en de opperhuid van meelwormen als substraat toegevoegd aan twee typen bodems. Om de totale bacterie gemeenschap en de chitinase bevattende chitinolytische bacterie gemeenschap te identificeren, heb ik met behulp van pyrosequencing het 16S rRNA en het catalytische domein van het bacteriële *chiA* gen geanalyseerd. Zowel de samenstelling van de totale bacteriële gemeenschap en van de chitinolytische gemeenschap werden significant bepaald door het bodem type, de aard van het toegevoegde chitine materiaal en de incubatie tijd. Echter, de diversiteit van de chitinolytische bacteriële gemeenschap was niet verschillend voor de behandelingen met toevoegingen van verschillende chitine bevattende materialen wat een aanwijzing is dat het chitine gehalte van het materiaal (dat

aanzienlijk verschilde per soort materiaal) geen groot effect heeft op de betrokken gemeenschap zo lang de chitine dezelfde structuur heeft.

Ik heb ook de *chiA* gen diversiteit en hoeveelheid als ook de dynamiek van de chitinolytische bacteriële gemeenschap in respons op de dynamiek van de saprotrofische schimmel biomassa in de rhizosfeer van aardappelen getoetst om zo de functie van bacteriële chitinases als agens in de competitie van bacteriën met schimmels te toetsen. De resultaten van dit onderzoek lieten zien dat een toename van de schimmel biomassa in de rhizosfeer van aardappelen leidde tot een toename van de *chiA* gen copy aantallen, een relatieve toename van *β*- en *γ*-*Proteobacteria* en tot een afname van het aantal *Acidobacteria*, wat erop duidt dat chitinases inderdaad een rol spelen in de interacties tussen bacteriën en schimmels in de bodem.

De resultaten van dit onderzoek hebben bijgedragen tot een beter begrip van de ecologische functies van chitinases. Nieuwe inzichten zijn verkregen over de samenstelling van het bacteriële chitinolytisch systeem en tot het belang van de componenten van dat systeem. De potentiele ecologische functies van het bacteriële chitinase complex zijn verder geëxploreerd en de rol van chitinases in bacterie-schimmel interacties , die vitaal zijn voor het functioneren van terrestrische ecosystemen, zijn verder aan het licht gebracht.

摘要

几丁质是自然界第二大产量的天然化学物质。在水生生态系统，几丁质主要来源于节肢动物的外壳，在土壤中几丁质主要来源于真菌细胞壁。能够降解几丁质的酶被成为几丁质酶。真菌和细菌都会产生这种酶。土壤中的细菌几丁质酶可以降解真菌菌丝以及其他几丁质，同时由于它们可以通过降解几丁质抑制活的真菌生长，因此含有几丁质酶的细菌也具有对真菌的拮抗功能。这篇博士论文的主要目标是测试以下假设：细菌几丁质酶是否在不同的真菌环境中会发挥不同的功能，以及在不同环境中细菌几丁质酶的基因组成和主要功能是否有区别。

在此研究中，我应用了不同的研究方法，包括生物信息学范畴的基因组比对和分子生物学的实验方法。在第二章中，来自陆生和水生环境含有几丁质酶的细菌基因组对比结果显示陆生细菌含有较为复杂的几丁质酶功能系统，这个结论符合我们的假设。比起水生环境，陆生环境的真菌含量较高，因此细菌几丁质酶的功能包括了降解含有几丁质的物质，同时也有对真菌的拮抗作用。对应的，我们观察得到了陆地细菌几丁质酶系统有着更为多样化的结合区域。另外，在陆地含有几丁质酶的细菌中，我们也观察到了更高的含有几丁质结合蛋白和可能参与针对真菌的拮抗作用的其他蛋白的可能性。

在第三章中，我们进行了一个实验来比对了 13 株不同的细菌对不同几丁质的降解能力和它们对真菌的拮抗作用。这 13 株细菌具有不同的几丁质酶数量，几丁质结合蛋白的数量，而且其他的形态属性也各不相同。令人惊异的是我们发现几丁质结合蛋白的数量与细菌降解晶体几丁质的能力成正相关。而几丁质酶的数量和细菌形态属性（有菌丝和无菌丝）没有影响几丁质的降解和细菌对真菌的拮抗作用。这个实验确定了几丁质结合蛋白在晶体几丁质降解过程中的重要作用。

为了研究在土壤中不同几丁质降解过程中对细菌群的选择，我在两种不同的土壤中添加了四种不同的几丁质，包括晶体状几丁质，两种真菌细胞壁，

以及粉虫的含几丁质外壳。然后我通过对细菌 16S rRNA 高通量测序的方法研究了降解过程中的细菌菌群，并通过对 *chiA* 基因的接触区间的高通量测序来鉴定几丁质的降解菌群。结果表明土壤、几丁质来源和添加时间都对菌群组成有着显著的影响。但是，菌群的丰度和多样性并不受几丁质来源的影响。

为了验证细菌几丁质酶在与真菌竞争中的作用，我还测试了 *chiA* 基因的丰度和多样性与马铃薯根际真菌数量的关系。结果表明马铃薯根际真菌数量的增加会导致 *chiA* 基因的数量增加；beta- and gamma- *Proteobacteria* 在土壤菌群中的相对丰度的增加，*Actinobacteria* 的丰度减少。这些结果证明了在土壤细菌真菌相互作用中细菌几丁质酶的确有一定的作用。

当前研究促进了对细菌几丁质酶生态功能的理解，揭示了细菌几丁质酶系统的重要性和组成的新内容，在对陆地生态系统中及其重要的细菌真菌相互作用中细菌几丁质酶体系的作用也有了进一步的研究。

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Supplementary tables

Supplementary table 1 Composition (%) of *chiA* coding bacterial community in genus level from buried nylon bags containing pure sand amended with different chitin resources. Bags were buried in grassland soil or forest soil. (CHI: crystal chitin; MUC: *Mucor hiemalis* cell wall; ASP: *Aspergillus niger* cell wall; MEA: mealworm cuticle). Identification is based on 16S rDNA sequences (ENA accession number: PRJEB9706).

	Grassland soil								Forest soil							
	First harvest				Second harvest				First harvest				Second harvest			
Family	CHI	MUC	ASP	MEA	CHI	MUC	ASP	MEA	CHI	MUC	ASP	MEA	CHI	MUC	ASP	MEA
Actinomycetaceae	2	0	8	3	11	1	5	3	23	1	1	1	40	1	7	22
Armatimonadaceae	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Bacteroidaceae	39	28	3	8	12	14	4	4	0	13	0	0	0	0	0	0
Chlamydiae	0	0	0	1	1	0	1	2	0	0	0	0	0	0	0	0
Opitutaceae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Unclassified Spartobacteria	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Acidobacteriaceae	0	1	0	0	0	0	0	0	2	4	6	3	21	25	66	8
Planococcaceae	0	0	0	10	0	0	0	17	0	0	0	12	1	0	0	33
Peptostreptococcaceae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Gemmatimonadaceae	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0
Planctomycetaceae	0	0	0	0	3	3	0	3	0	0	0	0	0	0	0	0
Caulobacteraceae	1	7	0	1	2	14	4	3	2	6	1	4	1	29	1	1
Bradyrhizobiaceae	4	1	0	1	1	2	1	1	0	0	0	0	0	0	0	0
Brucellaceae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Hyphomicrobiaceae	0	0	0	1	2	2	1	4	0	0	0	0	0	0	0	0

Supplementary tables

	Grassland soil								Forest soil							
	First harvest				Second harvest				First harvest				Second harvest			
Family	CHI	MUC	ASP	MEA	CHI	MUC	ASP	MEA	CHI	MUC	ASP	MEA	CHI	MUC	ASP	MEA
Methylobacteriaceae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Phyllobacteriaceae	2	1	1	1	4	2	1	4	1	0	0	7	0	0	0	0
Rhizobiaceae	6	1	3	5	10	6	5	3	9	1	0	0	1	1	0	0
Rhodobiaceae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Xanthobacteraceae	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0
Acetobacteraceae	0	0	0	0	0	0	0	0	0	1	0	0	1	2	2	0
Rhodospirillaceae	0	1	0	0	0	1	1	0	2	3	0	3	0	3	0	0
Rickettsiaceae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sphingomonadaceae	1	2	0	4	21	13	7	9	1	1	0	0	0	0	0	0
Alcaligenaceae	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	1
Burkholderiaceae	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0
Comamonadaceae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Oxalobacteraceae	10	6	1	3	5	5	0	1	0	1	0	0	0	0	0	0
Neisseriaceae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bacteriovoracaceae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bdellovibrionaceae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Myxococcaceae	0	0	1	1	0	1	1	2	0	0	0	0	0	0	0	0
Nannocystaceae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Polyangiaceae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Aeromonadaceae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Enterobacteriaceae	0	0	7	1	0	0	18	1	3	2	79	9	1	0	8	1
Coxiellaceae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

	Grassland soil								Forest soil							
	First harvest				Second harvest				First harvest				Second harvest			
Family	CHI	MUC	ASP	MEA	CHI	MUC	ASP	MEA	CHI	MUC	ASP	MEA	CHI	MUC	ASP	MEA
Legionellaceae	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Pseudomonadaceae	33	46	20	35	20	28	15	22	56	58	9	47	18	9	1	8
Thiotrichales	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sinobacteraceae	0	3	0	13	3	4	1	17	1	8	1	8	4	26	6	14
Xanthomonadaceae	1	0	54	10	1	2	36	4	1	1	1	2	12	2	7	10
Thermoproteaceae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Supplementary tables

Supplementary table 2 Number of chitinolytic bacteria isolated from buried nylon bags containing pure sand amended with different chitin resources. Bags were buried in grassland soil or forest soil. (CHI: crystal chitin; MUC: *Mucor hiemalis* cell wall; ASP: *Aspergillus niger* cell wall; MEA: mealworm cuticle). Identification is based on 16S rDNA sequences (Genbank: KT005619-KT005773).

	Grassland soil								Forest soil							
	First harvest				Second harvest				First harvest				Second harvest			
Genus	CHI	MUC	ASP	MEA	CHI	MUC	ASP	MEA	CHI	MUC	ASP	MEA	CHI	MUC	ASP	MEA
Flavobacterium	4	3	2	3	8	5	0	0	0	0	0	0	0	0	0	0
Chitinophaga	3	1	0	3	0	1	0	0	0	0	0	0	0	0	0	0
Pseudomonas	3	0	1	1	3	2	0	4	0	0	0	1	0	0	0	1
Unclassified *Oxalobacteraceae*	0	0	1	0	2	0	0	3	0	0	0	0	0	0	0	0
Stenotrophomonas	0	0	1	0	1	0	2	1	0	0	0	0	0	0	0	0
Rhizobium	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Variovorax	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Burkholderia	0	0	0	1	3	0	0	1	1	0	0	0	0	0	0	0
Paenibacillus	0	0	0	0	1	1	0	5	4	0	0	0	8	5	0	23
Janthinobacterium	0	0	0	0	4	1	0	6	5	2	0	0	0	0	0	1
Serratia	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	0
Luteibacter	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0
Collimonas	0	0	0	0	0	0	0	0	0	3	0	1	1	0	0	0
Dyella	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Bacillus	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	4
Leifsonia	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Pedobacter	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0
Herbaspirillum	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0
Total number	10	4	5	10	24	11	4	20	11	7	0	3	10	6	0	30

Supplementary table 3 Composition (%) of *chiA* coding bacterial community in genus level from buried nylon bags containing pure sand amended with different chitin resources. Bags were buried in grassland soil or forest soil. (CHI: crystal chitin; MUC: *Mucor hiemalis* cell wall; ASP: *Aspergillus niger* cell wall; MEA: mealworm cuticle). Identification is based on 16S rDNA sequences (ENA accession number: PRJEB9708).

	Grassland soil								Forest soil							
	First harvest				Second harvest				First harvest				Second harvest			
Genus	CHI	MUC	ASP	MEA	CHI	MUC	ASP	MEA	CHI	MUC	ASP	MEA	CHI	MUC	ASP	MEA
Janthinobacterium sp.	78	71	24	63	72	38	0	80	14	25	0	7	11	2	0	5
Stenotrophomonas	1	21	56	28	6	16	0	11	24	35	0	62	23	51	0	14
Panibacillus	0	0	0	2	4	3	0	3	0	0	0	0	48	14	0	19
Streptomyces	5	4	15	4	3	24	0	2	57	0	0	10	10	12	0	45
Rhodanobacter	0	0	0	0	0	0	0	0	0	32	0	10	1	8	0	4
Serretia	0	0	3	0	0	0	0	1	0	3	0	6	0	0	0	2
Kitasatospora	1	0	0	0	0	0	0	0	0	0	0	1	0	6	0	5
Burkholderia	9	2	0	0	5	10	0	0	4	2	0	2	1	0	0	3
Kutzneria	2	0	0	0	1	0	0	0	0	1	0	0	0	6	0	0
Bacillus	0	0	0	0	0	0	0	0	1	0	0	1	4	0	0	2
Cellvibrio	0	0	0	0	3	1	0	0	0	0	0	0	0	0	0	0
Arthrobacter	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0
Acidobacteriaceae	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
Amycolatopsis	1	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0
Chondromyces	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0
Chromobacterium	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0

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Yani Bai

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Curriculum Vitae

Yani Bai was born at April 16th 1983 at Yijun, China. In 2010, she received her master degree at Northwest Agriculture and Forest University, Yangling, Shaanxi Province, China. During her master study, her major was on Resource and Environmental Biology, specifically environmental microbiology; the title of her master thesis was “A Study of bacterial community on Amendment of Saline-alkali Soil by Addition of Sulfur”. In her master thesis she focused on bacteria that can utilize sulfur to decrease the pH of saline-alkaline soils in northwest China. Since September 2010, she started her PhD project at Netherlands Institute of Ecology (NIOO-KNAW), under supervision of Prof. Dr. Hans van Veen and Prof. Wietse de Boer. The results of her research are described in this thesis.